STUDIES ON OVINE INTERLEUKIN-1

by

Carolyn Ewen Fiskerstrand
B.Sc.Hons (Rand)

This thesis is submitted as part of the course requirements for the degree of Doctor of Philosophy at the University of Edinburgh
1993

UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 3.5.10)

Name of Candidate Carolyn Ewen Fiskerstrand

Address Department of Veterinary Pathology, (RDSVS), Summerhall, Edbgh.

Degree Doctor of Philosophy *Date* August 1993

Title of Thesis Studies on Ovine Interleukin-1

No. of words in the main text of Thesis 86,000

Interleukin-1 (IL-1) is a key mediator of infection, inflammation and immunity and two different IL-1 proteins are known to exist, IL-1α and IL-1β. Each is synthesised as a proprotein, M_r = 31kD, which is subsequent cleaved to yield the mature protein, M_r = 17.5kD. Whereas both forms of IL-1α show equivalent biological activities, IL-1β requires cleavage with resultant conformational change for optimal activity. IL-1 has been extensively studied in human and murine systems but at the time this project was initiated, nothing was known about its actions in the sheep and no reagents were available with which to study ovine IL-1. This thesis describes the successful cloning and expression of biologically active ovine IL-1α and IL-1β and their use in determining IL-1 receptor (IL-1R) expression by ovine alveolar macrophages (Mϕ) and afferent lymph dendritic cells (DC).

Lipopolysaccharide (LPS) stimulated Mϕ were used as the source of IL-1 mRNA. The specific IL-1 cDNAs were amplified by polymerase chain reaction, cloned into pTZ18R/19R vectors and sequenced. Ovine IL-1α and IL-1β were found to be 97% and 96% identical to their bovine counterparts and 81% and 78% identical, respectively, to the human sequences. Translation of the nucleotide sequences shows that ovine IL-1α has 97% and 72% identity with bovine and human IL-1α respectively and ovine IL-1β has 95% and 62% identity with bovine and human IL-1β respectively. Use of the cloned IL-1cDNAs for northern blot analysis of IL-1 mRNA production by Mϕ, showed IL-1α mRNA to reach maximal levels at around 6h and IL-1β mRNA at around 4h after LPS stimulation.

The proprotein (p) and mature protein (m) forms of ovine IL-1α and IL-1β have been expressed as fusion proteins with yeast p1, using the Ty-vlp system of British Biotechnology Ltd. The fusion protein self-assembles in the form of a virus-like particle (vlp), from which the rIL-1 is cleaved by the action of Factor Xa restriction protease (FXa). FXa cleavage of IL-1αp:p1 vlps revealed a relaxed FXa recognition site within IL-1αp which yielded a putative N-terminus at Ser-120, equivalent to the N-terminus of human IL-1αm. It is proposed that FXa may be one of the natural processing enzymes for IL-1α. The activities of purified IL-1 mature proteins on ovine thymocytes were ~2.5×10^7 U/mg IL-1α and ≥1.25×10^6 U/mg IL-1β. Activities on ovine xiphoid cartilage were ten times less. Ovine rIL-1 shows a species preference for homologous thymocytes and cross-species sequence analysis highlights 5 amino acid residues which may be of relevance in this context. Ovine rIL-1βp is biologically active, but about 5 times less so than IL-1βm.

By using ovine ^{125}I-rIL-1, ~16500 IL-1R/cell (Kd ~4.6pM) for IL-1β and ~2600 IL-1R/cell (Kd ~56pM) for IL-1α were detected on Mϕ. ≤24% of Mϕ expressed IL-1R. DC, isolated from lymph from cannulated sheep pseudoafferent lymph ducts, expressed ~510 IL-1R/cell (Kd ~30pM) for IL-1α and ~350 IL-1R/cell (Kd ~160pM) for IL-1β. Quantitation of IL-1R expressed by fresh DC has not previously been reported for any species, as far as I am aware. Following secondary ovalbumin challenge of primed sheep, DC expression of receptors for IL-1α, but not for IL-1β, is greatly upregulated. Two peaks of increased expression were detected, a transient increase at around 4h followed by an increase to maximal expression of ≥21700 IL-1α sites/cell (Kd ~181pM) between 43-72h. Only a very small proportion of individual DC were seen to bind IL-1 even after secondary antigen challenge.

DECLARATION

I hereby declare that the composition of this thesis and the work presented herein are my own, except where specifically stated in the text. No part of this work has been, or is being, submitted for any other degree or qualification

Carolyn Ewen Fiskerstrand
August 1993

For my Parents

with thanks

ACKNOWLEDGEMENTS

I am indebted to numerous colleagues for discussions, advice and assistance during the work presented in this thesis. In particular, I would like to thank Professor Ian McConnell for the opportunity of carrying out these studies within the Department of Veterinary Pathology, Dr. David Sargan, whose supervision has been invaluable, both during the experimental period and in the preparation of this manuscript, Dr. Douglas Roy for much appreciated help with protein expression work and Dr. John Hopkins for surgical work on sheep, without which the receptor expression data could not have been obtained.

The components of the Ty-vlp expression system were kindly supplied by Dr. Sally Adams of British Biotechnology Ltd., porcine IL-1β by Dr. J Saklatvala of the Strangeways Laboratories in Cambridge and anti-ovine monoclonal antibodies by various members of the Department of Vet. Pathology who are credited where appropriate in the text. The work was supported by a Wellcome Trust development award and grants from the Agriculture and Food Research Council and the Scottish Home and Health Department, to all of whom I am very grateful.

I very much appreciate the support, encouragement, tolerance and humour of my parents and of friends, especially Dinah Thom, David Christie and Christopher Baines, which has helped me survive the last few years of study.

INDEX

LIST OF FIGURES **Page**

Chapter 5

LIST OF TABLES **Page**

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
Ag	antigen
αm	ovine rIL-1α mature protein
αm:P1	ovine rIL-1α mature protein:P1 fusion protein (vlp)
AP	ammonium persulphate
αp	ovine rIL-1α preprotein
αp:P1	ovine rIL-1α proprotein:P1 fusion protein (vlp)
β-ME	2-mercaptoethanol
BCIP	bromo-chloro-imidolyl-phosphate toluidine salt
bFGF	basic fibroblast growth fctor
βm	ovine rIL-1β mature protein
βm:P1	ovine rIL-1β mature protein:P1 fusion protein (vlp)
bo	bovine
βp	ovine rIL-1β proprotein
bp	base pair
βp:P1	ovine rIL-1β proprotein:P1 fusion protein (vlp)
BSA	bovine serum albumin
cAMP	cyclic AMP
C_f	final concentration
CFA	complete Freunds' adjuvant
cGMP	cyclic guanosine monophosphate
CHAPS	(3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate)
ConA	concanavalin A
CSF	colony stimulating factor
CSF	colony-stimulating factor
DC	dendritic cell
DEX	dexamethasone
DMSO	dimethylsulphoxide
dNTP	deoxyribonucleotides
DOC	NaDeoxycholate
DTT	dithiothreitol
E. coli	*Escherischia coli*
EBV	Epstein Barr virus
EDTA	ethylenediaminetetraacetic acid
ELAM-1	endothelial leucocyte adhesion molecule-1
FACS	fluorescence activated cell sort analysis
FcR	cell-surface receptor for the Fc fragment of immunoglobulin
FCS	fetal calf serum
ϕGF	fibroblast growth factor
FGF	fibroblast growth factor
FITC	fluorescein
FXa	activated coagulation Factor Xa restriction protease
G-CSF	granulocyte-colony stimulating factor

GM-CSF	granulocyte-macrophage-colony stimulating factor
GTC	guanidinium *iso* thiocyanate
HBSS	Hanks balanced salt solution
HPLC	high pressure liquid chromatography
hu	human
ICAM-1	intercellular adhesion molecule-1
ICE	interleukin-1 converting enzyme
IDDM	insulin-dependent diabetes mellitus
IFA	incomplete Freunds' adjuvant
IFN	interferon
IL-	interleukin-
IL-1α	interleukin-1 alpha
IL-1β	interleukin-1 beta
IL-1ra	IL-1 receptor antagonist
IL-1RI	IL-1 receptor type I
IL-1RII	IL-1 receptor type II
IPTG	isopropyl-β-thiogalactoside
IRAP	IL-1 receptor antagonist protein
k/kD/kDa	kilodaltons
kb	kilobases
K_d	dissociation constant
LB	Luria-Bertani broth
LC	Langerhans' cell (epithelial)
LFA-1	leucocyte function antigen
LIF	leukemia inhibitory factor
LPS	gram-negative bacterial lipopolysaccharide
M-CSF	macrophage colony stimulating factor
Mϕ	macrophage
MHC	major histocomatibility complex
MLR	mixed leukocyte reaction
MNL	mononuclear leukocytes - refers to monocytes within the context of this thesis, unless otherwise stated
mu	murine
NBT	nitroblue tetrazolium
NF-	nuclear factor
NK	natural killer (cells)
OA	osteoarthritis
ov	ovine
p	porcine
P-A axis	pituitary-adrenal axis
p1	yeast p1 protein constitutively expressed from pMA5620 plasmid
PAGE	polyacrylamide gel electrophoresis
PBA	PBS containing 1mg/ml bovine serum albumin and 0.1% Na azide
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PGE_2	prostaglandin

PHA	phythemagglutinin (lectin)
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol myristyl acetate
PMN	polymorphonuclear leukocytes
RA	rheumatoid arthritis
rab	rabbit
RIA	radioimmunoassay
rProtein	recombinant protein
S. cerevisiae	*Saccharomyces cerevisiae*
SLE	systemic lupus erythematosis
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
TE	tris-HCL-EDTA
TGF	transforming growth factor
Th_1/Th_2	T helper cell subtypes
TNF	tumour necrosis factor
TYα	pOGS40 / IL-1α expression construct
TYβ	pOGS40 / IL-1β expression construct
VCAM-1	vascular cell adhesion molecule-1
vlp	virus-like particle
x-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Single-letter codes and abbreviations for amino acids

A	alanine (Ala)
C	cysteine (Cys)
D	aspartic acid (Asp)
E	glutamic acid (Glu)
F	phenylalanine (Phe)
G	glycine (Gly)
H	histidine (His)
I	isoleucine (Ile)
K	lysine (Lys)
L	leucine (Leu)
M	methionine (Met)
N	asparagine (Asn)
P	proline (Pro)
Q	glutamine (Gln)
R	arginine (Arg)
S	serine (Ser)
T	threonine (Thr)
V	valine (Val)
W	tryptophan (Try)
Y	tyrosine (Tyr)

CHAPTER 1

INTRODUCTION

1.1 Historical Perspective

Interleukin-1 was originally isolated from neutrophils by Menkin and Beeson in the 1940s as a fever-inducing substance called granulocyte pyrogen (GP). Subsequently other factors were isolated, endogenous pyrogen (EP) from blood of rabbits made fibrile by injection of bacteria, leukocyte endogenous mediator (LEM) which was thought to induce acute phase responses, and lymphocyte activating factor (LAF) which described a factor derived from mononuclear phagocytes which enhanced mitogen-driven thymocyte proliferation. It was not until 1972 that lipopolysaccharide (LPS) stimulated monocytes rather than neutrophils were found to be the major source of EP. Around the same time it was beginning to be realised that agents stimulating EP release also stimulated LAF production, the kinetics of release being similar (Atkins et al., 1960; Gery et al., 1971,1972; Gery and Waksman, 1972; Wolff, 1973; Kampschmidt, 1981). On purification, LPS stimulated monocyte supernatants yielded active protein products of heterogeneous molecular weight (12000 - 17000) with a broad spectrum of isoelectric points (pI 5 - 7). Two forms of human IL-1 of differing pI were subsequently resolved, IL-1α (pI 5) and IL-1β (pI 7) (Auron et al., 1984; Lomedico et al., 1984; Furutani et al., 1985; March et al., 1985), and equivalent proteins have now been identified in a number of species.

The term "Interleukin" was coined in 1979 at the Second International Lymphokine Workshop in Ermatingen, Switzerland and was primarily devised to describe the soluble mediators produced by activated T-lymphocytes which acted at short range on responder lymphocytes. It was decided that the 'monokine' variously referred to as GP, EP, LEM, and LAF should henceforth be known as Interleukin-1 (IL-1) (Mizel and Farrar, 1979). Other activities now attributed to IL-1 are those previously known as mononuclear cell factor (Krane et al., 1985), catabolin (Saklatvala et al., 1983), osteoclast-activating factor (Dewhirst et al., 1985) and hemopoetin-1 (Moore and Warren, 1987; Mochizuki et al., 1987).

Is is now clear that although the actions of IL-1 are extremely diverse, the main aspects of its function are immunostimulation, mediation of shock and inflammation, stimulation of hematopoesis and tissue moulding.

1.2 Sources of IL-1

IL-1 is now known to be produced by the majority of mammalian cell types, with the production of IL-1α and IL-1β being differentially regulated, presumably a consequence of functional requirements. Certain cells appear to produce either IL-1α or IL-1β virtually exclusively. With the exception of a few cell types, notably keratinocytes and certain central nervous system cells, IL-1 mRNA only becomes detectable in response to various stimuli. High levels of IL-1 expression are seen in the spleen, liver and kidney of normal individuals (Tovey et al., 1988). Proteins with IL-1-like activities have been detected in invertebrates (Beck and Habicht, 1986) but these will not be addressed further.

Both forms of IL-1 are initially produced as a proprotein of about M_r=31K which is subsequently cleaved to a mature M_r=17-18K form (Sect 1.4). The proprotein and mature forms are equally active. IL-1α is predominantly cell associated with small quantities only of the mature form being secreted (Conlon et al., 1987; Fuhlbrigge et al., 1988). Pro-IL-1α is glycosylated and about 10% is known to be membrane bound via a lectin-like interaction which is specifically dissociated with D-mannose (Brody and Durum, 1989). On the other hand IL-1β in all species studied so far is secreted and appears to require cleavage and consequent conformational change for both receptor binding and activity (Mosley et al., 1987a; Schmidt and Bomford, 1991). Certain recombinant pro-IL-1βs are reportedly active as will be detailed in Sect. 1.9. A small proportion of IL-1β may also be membrane bound (Folks et al., 1987; Hawrylowicz et al., 1989a).

Epithelial cells, fibroblasts and polymorphonuclear leucocytes all produce IL-1, the latter very inefficiently, (Wagner et al., 1985; Bandari et al., 1989; Canning and Neill, 1989). Endothelial and smooth muscle cells express biologically active IL-1 on their surface but do not release it in any quantity and activated platelets produce IL-1α and IL-1β which are both entirely membrane bound (Hawrylowicz et al., 1989a). IL-1β but no IL-1α has been detected from synovial fibroblasts and in the stromal areas of ovarian cancer cells (Bandari et al., 1989; Naylor et al., 1990). Only IL-1α is synthesised by human mesangial cells (Abbott et al., 1992).

Keratinocytes and sweat gland epithelium (Luger et al., 1991; Reitamo et al., 1990) constitutively produce IL-1 thus creating a major reservoir of IL-1 in the skin. Unlike cultured keratinocytes which produce active IL-1α precursor molecule and accumulate large amounts of inactive, unprocessed IL-1β precursor (Bigler et al., 1992), both normal and psoriatic keratinocytes do process IL-1β to a mature form, although somewhat ineffectively (Cooper et al., 1990a; Hammerberg et al., 1990). This epidermal form of IL-1β has been shown to be incapable of stimulating cells possessing the type I IL-1 receptor (Cooper et al., 1990b). U937, a human histiocyte derived cell line, produces proteins analogous to the inactive precursors of both IL-1β and TNFα but the active forms of either have not yet been detected (Demeter et al., 1991).

Of the inducible IL-1 producers, stimulated monocytes, tissue macrophages and glial cells are the major sources of IL-1, with generally more IL-1β than IL-1α being produced. B cell production of IL-1 differs significantly from that of macrophages both in kinetics and in the signals which induce it. TNF and LPS, which are both potent stimulators of Mϕ IL-1, have no effect on B cells, whereas IL-2 and IL-4 both stimulate B-cell but not Mϕ IL-1 production. B cell IL-1 expression is several orders of magnitude less than that of Mϕ and is significantly slower, peaking at about 48h compared to less than 6h after Mϕ stimulation (Hawrylowicz et al., 1989b; Weaver and Unanue, 1986). Constitutive IL-1 production from normal cultured human B lymphocytes (Pistoia et al., 1986), can be increased by stimulatory agents.

Blood dendritic cells do not produce detectable IL-1 (Bhardwaj et al., 1989; Vakkila et al., 1990). Dendritic cells and their relationship to IL-1 will be dealt with in Sect. 1.15.2.

There is much evidence for disconnected synthesis and secretion of IL-1α and β. Studies on connective tissue diseases show increased concentrations of extracellular IL-1α and β but intracellular concentrations remain normal and do not correlate with their respective extracellular concentrations (Aotsuka et al., 1991). Whilst cultured bone marrow-derived macrophages remain able to produce IL-1, IL-6 and TNFα over a 3-week period, their ability to release IL-1 is lost although the intracellular accumulation is unchanged (Aznar et al., 1990). Sheep red blood cells and carbonyl-iron, but not the compounds hemoglobin, globin and ferric citrate, enhance IL-1β production from phagocytic, but not non-phagocytic, human blood monocytes indicating that phagocytosis must be an important trigger for this mechanism of IL-1 release (Okamoto et al., 1991).

Studies on inflammatory sites have yielded much information on the production and involvement of cytokines in tissue function, see Sect. 1.5. LPS induced

release of IL-1 from peritoneal macrophages during the exudative response to Freund's complete adjuvant, over 30days, shows that IL-1 is produced at high levels much earlier than TNF and is associated with the recruitment phase of the reaction (Chensue et al., 1989). TNF seems to be associated more with the established exudate stage. Immunohistochemically, IL-1α and β show comparable expression at 6h post LPS but whereas IL-1α persists in the cytoplasm for at least 18h, IL-1β rapidly disappears from the adjuvant recruited cells.

1.3 Induction of IL-1 - Transcriptional and Translational Control

The term 'Cytokine Network' refers to the overlapping functions of two or more cytokines which can have synergistic effects or may antagonise each other. In addition, the production and release of cytokines are influenced by positive and negative feedback loops from the same or other cytokines. External agents can also initiate cytokine synthesis and interactions. As an example, bacterial endotoxin (LPS) induces primarily IL-1β and TNF synthesis from macrophages. In response to these two cytokines, IL-6 and IL-8 are produced which, in turn, inhibit the production of IL-1 and TNF.

The most commonly used stimulus for IL-1 transcription is LPS but other agents which induce factors required for IL-1 gene transcription include complement components, clotting components, bile salts and the cytokines TNFα, CSF-1, GM-CSF and IL-1 itself. Inhibitory agents include corticosteroids, prostacyclin, prostaglandins and cytokines IL-4, IL-6, IL-8, IL-10 and TGFβ. The precise pattern of IL-1 gene response, ie. enhanced and suppressed transcription, mRNA stability and translational control is dependent on the stimulus. IL-1α and IL-1β are under separate transcriptional control and transcription and translation of IL-1 are distinct and dissociated processes in macrophages (Mϕ), peripheral blood monocytes (PBMC) and polymorphonuclear leukocytes (PMN) (Marucha et al., 1991; Ohmori et al., 1990; Turner et al., 1989; Yamoto et al., 1989).

Macrophages are thought of as being the prime controllers of cytokine influences in normal and diseased tissues. The response to endotoxin (lipopolysaccharide, LPS) stimulation is dramatic. LPS and CSF-1 induce early genes encoding functional proteins eg. IL-1 and TNF as well as immediate early genes (*c-fos, c-myc, c-fms, c-fgr*) which initiate both positively and negatively interacting transduction cascades. Production of IL-1α, IL-1β, IL-6, TNFα and M-CSF from LPS stimulated monocytes and macrophages is differentially modulated by numerous factors. IL-4 is thought to be one of the major factors involved in regulation of IL-1 production (Wong et al., 1993). It inhibits

production of IL-1β and TNFα from LPS, IFNγ or IL-1α stimulated macrophages whilst acting synergistically to stimulate IL-1 receptor antagonist (IL-1ra, see Sect. 1.13.1) production (Vannier et al., 1992; Hart et al., 1991). Different IL-4 actions have been detected in different species (Hart et al., 1991).

Production of IL-1 from human monocytes in culture is affected by serum concentrations in the medium. Cells in medium with 1% heat-inactivated serum produced about 3-fold more IL-1α than IL-1β by 18h whereas 1% non-inactivated serum or 5% fresh plasma induced similar amounts of IL-1α and IL-1β. In all cases 80-90% of IL-1α remained cell associated for 24h with increasing amounts being found extracellularly subsequently (Lonnemann et al., 1989).

Cytokine release varies with the state of maturation of the monocytes. In tissue culture monocyte to macrophage differentiation shows M-CSF to be the only cytokine secreted constitutively up to 7 days and becomes inhibited by LPS. Of the LPS dependent cytokines, IL-1β and IL-6 are down regulated and TNFα increases greatly (Scheibenbogen and Andreesen, 1991). After LPS stimulation, more than 81% of monocytes but only about 43% of macrophages express IL-1 mRNA transcripts (Bernaudin et al., 1988).

Although LPS induces similar increases in mRNA levels in monocytes (MNL) and polymorphonuclear leukocytes (PMN), with IL-1β transcription predominating over IL-1α, mRNA translation in PMN is far less efficient. PNM must be subject to specific control because rabbit reticulocyte lysates will translate MNL and PMN IL-1 mRNAs equally efficiently (Lord et al., 1991).

Complement, β-glucan polymers and adherence of monocytes/macrophages to surfaces all induce transcription without translation and in each case the steady state levels for IL-1β mRNA are comparable to those detected after 10ng/ml endotoxin (Schindler et al., 1990b,c,d; Yamoto et al., 1989). C5a, which is produced during activation of the complement cascade and is a potent mediator of various inflammatory events, stimulates IL-1 release from monocytes and there is significant synergism with LPS or IFNγ (Okusawa et al., 1987). The signal provided by C5a is primarily transcriptional, the translational signal being provided by LPS or IL-1 itself (Schindler et al., 1990b,c; Geiger et al., 1992). IL-6 production can not be triggered by C5a alone but C5a significantly increases IL-6 production from LPS or IL-1 stimulated monocytes, indicating a potentially important differential regulation of IL-1 and IL-6 by C5a (Montz et al., 1991). IFNγ has no effect on either IL-1 or TNF mRNA in macrophages but does enhance cytokine synthesis from GM-CSF, TNFα or LPS stimulated cells. IFNγ increases and sustains LPS induction of IL-1α and β dramatically as

opposed to inducing only a transient TNFα response. On the other hand, IFNγ suppresses IL-1 transcription from IL-1 stimulated cells (Chantry et al., 1990; Schindler et al., 1990a; Ucla et al., 1990). IL-4, IL-6 and TGFβ suppress IL-1 transcription and IL-10 inhibits IL-1 expression from LPS stimulated monocytes (Hart et al., 1991; Schindler et al., 1990b; Chantry et al., 1990; de Waal Malefyt et al., 1991).

Cycloheximide, a translation inhibitor, added together with TNF superinduces IL-1β mRNA and stabilises both IL-1 mRNAs, indicating posttranscriptional control in monocytes (Turner et al., 1989). In PMN the effect is different and cycloheximide abrogates TNF, or IL-1 plus TNF, induced accumulation of IL-1β mRNA, an effect not caused by inhibition of IL-1β mRNA transcripton. During experimental endotoxemia, *in vivo* treatment with dexamethasone (DEX), an immuno-suppressive and anti-inflammatory agent, prevents LPS induction of IL-1α mRNA and suppresses but does not completely inhibit IL-1β mRNA transcription (Ulich et al., 1990). Glucocorticoids also selectively decrease both transcription and stability of IL-1β mRNA (Nishida et al., 1988b; Lee et al., 1988) Nonsteroidal anti-inflammatory drugs do not stimulate IL-1 production but relieve the inhibition caused by the presence of prostaglandins (Otterness et al., 1988). *In vivo* evidence supports the importance of lipoxygenase products in the reduction of IL-1 synthesis and the 13-lipoxygenase products are thought to be involved in the early events of transcription (Endres et al., 1989; Schade et al., 1991).

Because of the differences in ability of stimuli to affect transcription and mRNA stabilisation, it has been concluded that the effects may be directed, in part, through second messenger pathways. IL-1 gene expression in monocyte lineage cells is regulated by calmodulin dependent-kinase while TNF is not affected. Hurme (1987) has shown from studies using phorbol myristate acetate (PMA), an activator of PKC, that PKC activation alone is sufficient for IL-1β gene induction but additional signals, provided by LPS, are required for the activation of the IL-1α gene. In the presence of PMA and cycloheximide, transcription is not induced indicating that induction of transcription with PMA requires *de novo* protein synthesis. IL-1 production by activators of PKC is suppressed by IFNγ (Schindler et al., 1990a) and DEX decreases PMA induction of IL-1β mRNA in U937 cells. Recently tyrosine kinase activity was shown to be required for the PMA induced IL-1β expression and that this activity is independent of PMA induced AP-1 activity (Palkama et al., 1993).

The effects of cAMP on IL-1 synthesis seem to be dependent on the stimulus. PGE-induced suppression of IL-1 could be via the induction of cAMP, raised

levels of which inhibit LPS induced translation but not transcription of IL-1 mRNA (Knudsen et al., 1986; Hurme and Serkkola, 1991). However, conflicting studies (Cavaillon and Haeffner-Cavaillon, 1990) have shown that LPS induced PGE_2 is not involved in elevation of intracellular cAMP and the cAMP suppression may be independent of PGE_2 effects. IL-1β production has also been shown to be increased by oral cyclo-oxygenase inhibitors such as indomethacin, which blocks IL-1 induced PGE_2 *in vitro* (Endres et al., 1989; Takii et al., 1992). Human immunoglobulin (IgG-IV), suppresses IL-1 and TNFα production from rabbit peritoneal exudate cells stimulated with LPS, possibly as a result of Ig binding to the cells via Fc gamma receptors which in turn increases intracellular cAMP levels (Shimozato et al., 1990). Histamine, which also reduces LPS-induced IL-1 via a cAMP pathway, enhances IL-1 induced IL-1 synthesis and gene transcription (Vannier and Dinarello, 1991).

Early reports claimed no central role for calcium in the induction and release of human IL-1 (Newton, 1987; Schindler et al., 1990a) but calcium ionophore has now been found to stimulate a transient increase in IL-1 mRNA from mast cells and to have some positive effect on IL-1 at both transcriptional and posttranscriptional levels in monocytes and macrophages (Labadia et al., 1990; Suttles et al., 1990; Mahe et al., 1991).

IL-1 and TNFα are very similar mediators and IL-6 mediates many of the effects of both IL-1 and TNFα, eg. in fever induction and there are several examples of discoordinate expression of these cytokines. Increased levels of PGE_2/cAMP can downregulate TNF expression by reducing its transcription rate, whereas IL-1 mRNA is not affected and IL-6 mRNA can be elevated by cAMP (Bailly et al., 1990; Endres et al., 1991; Kunkel et al., 1988; Scales et al., 1989). Raised soluble cGMP levels suppress LPS stimulated IL-1 production while having no effect on TNFα (Endres et al., 1991).

A general principle appears to be that only those cells which possess high numbers of LPS receptors are efficient inducible producers of IL-1.

1.4 Processing and Secretion of IL-1

The primary IL-1 translation products are 31K precursors which are largely found intracellularly in the cytosol and can be associated with lysosomes and microtubules but not golgi apparatus (Baldari and Telford, 1989; Bakouche et al., 1987). Myristyl groups have been detected on both proproteins and are thought to be added posttranslationally. These groups may be utilised for IL-1/membrane associations (Bursten et al., 1988).

Most IL-1α remains in the cytosol but some of the precursor form becomes membrane associated (Beuscher et al., 1987; Brody and Durum, 1989). A calcium activated neutral protease, calpain, processes IL-1α to the mature form with amino terminal Ser-113 (Kobayashi et al., 1990; Carruth et al., 1991) but numerous active IL-1α sub-fragments are routinely detected in biological fluids and appear to be generated by digestion at trypsin-sensitive sites or by serine proteases (Cannon and Dinarello, 1985). Monocyte IL-1α remains cell-associated for the first 20 hours and has a half-life of 15 hours (Hazuda et al., 1988).

Unlike IL-1α, the IL-1β precursor must be cleaved for optimal activity. Processing and secretion of IL-1β are closely linked. Although monocytes and macrophage secrete IL-1β efficiently, contrary to endothelial cells, smooth muscle cells and fibroblasts, there is controversy about IL-1β release and processing by monocytes/macrophages. Intact pro-IL-1β has been observed as the predominant released form and thought to be processed in the extracellular space (Hazuda et al., 1988, 1990; Beuscher et al., 1990; Suttles et al., 1990), but there is also evidence that the proprotein is processed within the cell and that processing may be concurrent with secretion (Hogquist et al., 1991a,b; Thornberry et al., 1992; Rubartelli et al., 1993). Still other data suggests that cultured monocytes release more of the unprocessed form than do fresh monocytes and that release in culture is pH and temperature dependent (Arend et al., 1989; Rubartelli et al., 1993). Cell injury releases predominantly the proprotein (Hogquist et al., 1991a,b). Although processed IL-1β is only observed extracellularly, both the processing enzymes and the IL-1 proprotein are present in the cytosol. The amount of IL-1 released and processed also depends on the stimulatory agent, eg. LPS induced IL-1β is rapidly released and processed but IL-1β induced by IL-1α or IL-2 remains cell-associated (Dinarello et al., 1987; Numerof et al., 1990). The effects of IL-1 as the inducer may depend on its concentration (Manson et al., 1989). Interaction of immune complexes with Fc receptors may drive monocytes to release pre-existing intracellular IL-1β (Rubartelli et al., 1993).

IL-1 has no conventional secretory signal sequence and various routes of release have been proposed, such as exocytosis from vesicles, active transport by carrier proteins or cell death (Bakouche et al., 1987; Rubartelli et al., 1990; Hogquist et al., 1991a,b). Data from expression of recombinant IL-1β suggests that fusion to a heterologous leader sequence allows IL-1β to be translocated across the membrane of the endoplasmic reticulum and to be transported and secreted via the endocytic pathway (Baldani et al., 1987; Pecceu et al., 1991). There is a growing number of extracellular proteins which, like IL-1, lack N-terminal

hydrophobic signal peptides, have glycosylation sites which do not appear to be utilised and are secreted with slow kinetics from producer cells (see Sect. 1.7.2 re glycosylation of IL-1β). Transporter proteins exist, such as the prokaryotic HlyB and CyaB, yeast Ste6 and murine Mdr, whose expression appears to be restricted to specific tissues and substrates (Kuchler and Thorner, 1990). Very recently members of the ATP-driven transporter family of proteins, which resemble HlyB and CyaB and have been detected in mammalian cells, have been proposed as candidate IL-1 transporters (Kuchler and Thorner, 1990; Howard et al., 1991; Rubartelli et al., 1993).

The amino terminus of mature IL-1β is Ala-117 but other naturally occuring N-termini have been reported. In particular, a 22K partially cleaved product is found in monocyte supernatants. Processing of IL-1β after secretion is by any of several proteases released at inflammatory sites. Proteinase K has been shown to transform IL-1β to the active form which is proteinase K insensitive, as is IL-1α. Elastase, cathepsin G, collagenase, all of which are found in bronchoalveolar lavage fluid, as well as serine proteases and plasmin have been shown to release active mature protein through cleavage at sites other than Ala-117 (Auron et al., 1984; Black et al., 1988; Hazuda et al 1988, 1990; Mizutani et al., 1991a). Cells such as keratinocytes do not produce enzymes which process IL-1β and the serine protease, mast cell chymase, is thought to process the released IL-1β locally (Mizutani et al., 1991b). The significance of these small peptides is not yet understood.

The highly specific monocyte processing enzyme, IL-1 converting enzyme (ICE), cleaves at Asp:hydrophobic motifs to yield active protein (Howard et al., 199; Mosley et al., 1987a; Sleath et al., 1990). This convertase enzyme has been cloned and is a member of the cysteine protease family (Ceretti et al., 1992; Thornberry et al., 1992). The mRNA is detectable in a variety of cells and it has been suggested that the enzyme may have other substrates as well as IL-1β precursor (Cerretti et al., 1992). Mapping of the enzyme to chromosome 11q23, which is frequently involved in rearrangement in human cancer, raises the possibility that altered production of the protease contributes to some of these disease states. The murine form of the enzyme, located on murine chromosome 9, has been detected in mononuclear phagocyte and T lymphocyte cell lines as well as in spleen, heart, brain and adrenal glands (Nett et al., 1992). The critical function of this protease is indicated by the finding that cowpox virus encodes a highly specific ICE inhibitor which is necessary for the virus to suppress the host inflammatory response (Ray et al., 1992).

1.5 Actions of IL-1

Interleukin-1 is a key mediator in the cytokine network, affecting many pathophysiological cell and tissue functions. The effects of systemic injection of IL-1 include increases sodium excretion, hypotension, depressed myocardial function, leukopenia, thrombocytopenia, pulmonary congestion and tissue neutrophilic infiltration. IL-1 belongs to a group of cytokines, IL-1, TNFα and IL-6, which have multiple overlapping actions. The pleiotropic nature of IL-1 actions has been well documented and although the majority of biological effects attributable to IL-1 are associated interchangeably with IL-1α and IL-1β, there are notable exceptions to this.

T-cell proliferation is dependent on the secretion of autocrine growth factors. The involvement of IL-1 is now becoming clarified. IL-1 induces IL-2 in T cells to a small extent but acts synergistically with IL-6 leading to an IL-2 dependent pathway of proliferation (Kuhweide et al., 1990). Blocking the IL-2R eliminates IL-1 induced effects but does not affect IL-6/PHA induced proliferation.

IL-1 is involved in B cell maturation and proliferation in response to antigenic stimulation. Activated B cells participate in the immune response as accessory cells for T cell activation, not only by physically presenting antigen but also by releasing IL-1 (Pistoia et al., 1986). However, unlike macrophages, B-cell antigen presenting potential is not enhanced by IL-1 (Krieger et al., 1986). Synergism with IL-6 is equally important for B and T cell activation. IL-1 can also act indirectly via stimulation of T cell secretion of various colony stimulating factors and IFNs which can act synergistically with IL-1 to promote B cell proliferation (Caussy and Sauder, 1989). Cell surface expression of CD5, expressed by the majority of T cells and some B cells, has also been cited as regulating the binding of and responsiveness to IL-1 of these cells (Nishimura et al., 1988).

Whilst GM-CSF and IL-3 are the most important mediators of stem cell differentiation, IL-1 plays a supplementary role (Takaue et al., 1990; Shieh et al., 1991; Zucali et al., 1991; Santiago-Schwarz et al., 1992)

IL-2 is the most effective stimulator of natural killer (NK) cells. IL-1 can trigger modest activation of these cells and evidence from human and porcine studies shows NK activity to be augmented by IL-1α and IL-2 in a non-synergistic fashion. It is thought that IL-1 may act by influencing IL-2R expression. The actions of both IL-1 and IL-2 are inhibited by IL-4 (Ostensen et al., 1989; Knoblock and Canning, 1992). It has also been shown, however, that IL-4 induces NK activity activity in human lymphocytes pre-activated by IL-2

(Higuchi et al., 1989). IL-1ra strongly potentiates the stimulatory action of IL-2 on NK activity in the same way as IL-1 but as IL-4 stimulates production of IL-1ra, inhibition of an intermediate pathway by the latter, may be involved (Conti et al., 1991). IL-1 may also act by increasing binding of NK cells to tumour cells (Herman et al., 1985).

IL-1 is involved in the recruitment of cells to sites of inflammation or injury via various mechanisms. Intrapulmonary IL-1 mediates acute IgG immune complex induced alveolitis in the rat, the dramatic increases in IL-1 concentrations showing positive correlation with recruitment of neutrophils to the lung (Warren, 1991). Potentially fundamental differences in the requirements for cytokines in lung and dermal vascular injury after deposition of IgG immune complexes have now become apparent. TNFα and IL-1 are both found in the lung but only IL-1 in the dermis (Mulligan and Ward, 1992). IL-1 exerts its influence on PMN by initiating PMN migration during an acute inflammatory response and is the dominant signal for movement of PMN from bone marrow to local sites of accumulation (McIntyre et al., 1991; Cybulski et al., 1986) and induced neutrophil migration across IL-1-activated endothelium is abolished by GM-CSF (Yong and Linch, 1993). IL-1, TNF and C5a have all been shown to be actively involved *in vivo* in the mobilisation of PMN to inflammatory sites (Mason and Van Epps, 1989) but TNF, and not IL-1, appears to induce PMN migration through fibroblast layers by an alternative fibroblast-dependent mechanism (Morzycki and Issekutz, 1991). Normal bovine neutrophils are known to be responsive to recombinant bovine IL-1β. It appears to augment phagocyte oxidative metabolic responses to subsequent stimulation by microbial antigens (Canning and Baker, 1990).

Synovial cells stimulated by IL-1 produce factors selectively chemotactic for neutrophils, lymphocytes and monocytes which could potentially facilitate inflammatory arthritis (DeMarco et al., 1991). In addition, synovial fibroblasts secrete IL-1α, which activates chondrocytes directly and bFGF, which potentiates the activity of IL-1. The IL-1 induced release of another, as yet unclassified, factor acting on chondrocytes has elicited the idea of a new cytokine (Bandara et al., 1992).

Chondrocytes are responsive to IL-1 which triggers the expression of collagenase, caseinase, phospholipase and cyclooxygenase genes, increases release of proteoglycans (the basis of one biological assay for IL-1 and TNF), induces large amounts of IL-6 and inhibits plasminogen activator. The metalloproteinase production is not affected by any factor such as LPS, PMA, Con A or indomethacin which affect T-cell proliferation. TNF, on the other

hand, only decreases plasminogen activator and does not elicit production of either the metalloproteases or PGE_2 (Schnyder et al., 1987; Shinmei et al., 1989). IL-1 directly increases transcription of Types I, III and IV collagen (Kahn et al., 19*) but inhibits Type II collagen synthesis by a mechanism involving DNA regulatory sequences on the collagen gene (Chandrasekhar et al., 1990).

Heckman et al. (1993) have demonstrated that that the effects of IL-1 on dermal fibroblasts may be concentration dependent. At low IL-1 concentrations, chemotactic responsiveness was enhanced whereas higher concentrations were required to stimulated collagenase mRNA synthesis. Fibroblasts and endothelial cells are a potent sources of hematopoetic growth factors such as GM-CSF and G-CSF when stimulated by IL-1 or TNFα (Pober et al., 1987; Broudy et al., 1987) but are not themselves targets for these factors (Yong et al., 1991). TGFβ production is also stimulated (Phan et al., 1992). *In vitro* senescent endothelial cells no longer respond to exogenous growth factors and contain large amounts of IL-1α transcript which is not found to any extent in transformed endothelial cells. The proliferative lifespan of the cells can be extended by treatment with an IL-1α mRNA antisense oligodeoxynucleotide, indicating that IL-1α can regulate the dynamics of endothelial cell senescence (Maier et al., 1990).

Endothelial and smooth muscle cells express biologically active IL-1 on their surface and do not release it in any quantity. The resultant requirement for intercellular contact between vascular cells and infiltrating leukocytes could conceivably permit costimulation while limiting undue propagation of inflammatory effects under normal conditions (Loppnow and Libby, 1992). Both IL-6 and TNF are released from these cell types on stimulation with IL-1 which is obviously acting as a controlling signal. IL-1 and TNF inhibit endothelial cell growth but stimulate smooth muscle cell growth and IL-6, acting alone, has also been proposed as being important for smooth muscle cell proliferation. The mechanism may involve differential regulation of the heparin-binding (fibroblast) growth factor receptors, the expression of which correlates inversely with mitogenic effects (Sawada et al., 1990; Morimoto et al., 1991).

The effects of IL-1, TNF and other cytokines on leukocyte-endothelial adhesion has led to the discovery of several endothelial adhesion molecules (Cotran and Pober, 1990). Platelet-derived IL-1, which is entirely cell associated, stimulates cytokine production from endothelial cells as well as inducing selective adhesion molecule expression which is dependent on the source of the cells eg. vascular expression of ELAM-1 occurs on cells from cutaneous rather than noncutaneous sites sites. Although other cytokines probably play an enhancing role, platelet-delivered IL-1 appears to be the essential component for stimulation of

endothelial cells by platelets, as antisera to IL-1α and β completely block platelet induced responses. In conjunction with an increasing volume of data on platelet-endothelial cell interactions, this delivery of IL-1 to vascular endothelial cells following injury, implicates platelets as providers of an initiating signal for the inflammatory response (Hawrylowicz et al., 1991).

ICAM-1 (CD54) is upregulated by IL-1α but LFA-3 (CD2) is not (Swerlick et al., 1991). A further adhesion molecule, ELAM-1 is only very transiently expressed upon stimulation by IL-1α (Beekhuizen et al., 1991). IL-1, TNFα and IFNγ also induce expression of the adhesion molecule ICAM-1 on synovial fibroblasts and increase the number of cells expressing it. Of the three cytokines IL-1 has the least effect. Removal of the cytokines causes a rapid decrease in ICAM-1 expression. At the same time HLA class I antigen expression is slightly increased by all three cytokines but only IFNγ increases class II expression (Chin et al., 1990).

Two recent reports have also indicated a role for cell-associated IL-1β. Data from patients on continuous ambulatory peritoneal dialysis which show that inflammatory peritoneal monocytes have reduced ability to secrete IL-1, compared to autologous blood monocytes but are still inflammatory, have raised the possibility that an intracellular location may be important for utilisation of IL-1β *in vivo* (Hart et al., 1993). Both LPS and IL-1β stimulated human fetal microglial cells produce abundant levels of potent IL-1β which remains primarily cell associated (Lee et al. 1993).

Monocyte MHC Class I expression is not upregulated by IL-1 (Swerlick et al., 1991; Migita et al., 1991). IL-1α has been shown to be a potent inducer of the extremely unstable prostaglandin H sythetase enzyme (PGHs) in macrophages. It is thought that CSF-1 is the main regulator during differentiation of pro-monocyte to monocyte and monocyte to macrophage and that IL-1α, IL-1β, IFNα, IFNβ and perhaps IL-2 regulate the differentiation of macrophages (Bartolini et al., 1990; Peters et al., 1992). Monocytes/macrophages are the main controllers of the cytokine network and the role of IL-1 is to stimulate the release of numerous cytokines and growth factors (see Appendix 2 for examples of cytokine networking). IL-1 has been strongly implicated as an hematopoetic factor, synergising with IL-3 in particular, but also with GM-CSF, in stimulating growth and differentiation of hematopoetic stem cells from bone marrow (Mochizuki et al., 1987; Kobayashi et al., 1991).

Bone metabolism is dependent on differential cytokine release by the various cells involved. eg. Macrophages are known to be involved in the early stages of laying down of osteoblasts which are derived from stromal cell progenitors, as

are chondrocytes. They produce IL-1β but also present membrane-bound IL-1 directly to bone calvaria. Mast cells are present in excessive amounts in conditions like RA and osteoporosis where there is bone loss (Nishihara et al., 1989). Mast cells and basophils are induced by IL-1 to produce histamine (Subramanian and Bray, 1987), which can in turn enhance IL-1 induced IL-1 synthesis and gene transcription in macrophages (Vannier and Dinarello, 1991). IL-1β is the most active cytokine to be involved in bone resorption and inhibition of bone formation. TGFβ acts most strongly in opposition but IFNγ and calcitonin also inhibit the resorptive effects of IL-1 and TNF (Boyce et al., 1989; Gowen et al., 1990). The actions of IL-1 are inhibited by IL-1ra (Rosen et al, 1990). IL-6 can be produced from bone cultures and may prove to be important in the regulation of osteoclast development and stimulation of resorption at an early stage of osteoclast recruitment. Glucocorticoids predispose to osteoporosis, an action effected by IL-1β, and have a positive effect on osteoclasts.

A mutual antagonism exists between IL-1 as pro-inflammatory and glucocorticoids as anti-inflammatory mediators. Examples of this phenomenon are DEX induced alkaline phosphatase production from endothelial cells which can be completely inhibited by simultaneous incubation with IL-1, and IL-1 induced neutral metalloproteinase synthesis from endothelial cells or fibroblasts which is inhibited by DEX and glucocorticoids via involvement of the glucocorticoid receptor (DiBattista et al., 1991; Mulkins and Allison, 1987). Stromelysin is a tissue-degrading proteinase important in tissue-moulding processes such as in wound healing, inflammatory reactions, rheumatoid arthritis and tumour invasion. Frisch and Ruley (1987) have demonstrated that DEX can suppress IL-1 and phorbol ester induction of transcription from the stromelysin promoter.

IL-1 acting in the brain (Dinarello 1984; Tobler et al., 1984; Besedovsky et al., 1986; Brown et al., 1991) has a variety of biological effects including induction of fever, slow wave sleep and potent stimulation of the pituitary-adrenal (P-A) axis via release of corticotropin-releasing factor (CRF). Intravenous injection of of IL-1α and IL-1β appear to result in distinct responses with only IL-1β significantly increasing plasma levels of adrenocorticotropic hormone (Uehara et al., 1987). Induction of IL-1 in the brain rapidly suppresses peripheral immune responses via the CRF-mediated activation of both the pituitary-adrenal axis and the sympathetic nervous system (Sundar et al., 1991). In cultured hepatocytes, IL-6 has also been implicated as the mediator of IL-1 and TNF induced synthesis of the acute phase proteins, C-reactive protein and serum amyloid A (Yap et al., 1991). In the gut, a possible interaction between the immune and gastrointestinal

systems has been suggested. The involvement of IL-1 includes stimulation of PGE2 synthesis by the stomach, retardation of gastric emptying and actions as a cryoprotective and antisecretory agent (Saperas et al., 1990; Robert et al., 1991). IL-1 has numerous endocrinologic effects, including inhibition of thyrocyte function and prolactin release and induction of ACTH with resultant increases in steroid synthesis (Krogh- et al., 1991, Migita et al., 1991). A number of reports indicate IL-1 induced increase in phosphorylation of serine residues of the small heat shock protein hsp 27. (see IL-1 signal transduction in Sect 1.12.2). Threonine and tyrosine residues are not phoshorylated in either control or stimulated cells (Kaur et al., 1989).

Table 1.1 gives a generalised summary of the main categories of IL-1 actions.

TABLE 1.1

Summary of the Main Categories of IL-1 Function

IL-1 Function	Target Cell
Proinflammatory	Monocyte/Macrophage
	Fibroblast
	Hepatocyte
	Endothelium
	Osteoblast
	Pancreatic islets
Mobilisation to inflammatory sites	Neutrophil
Degranulation and histamine release	Mast cell/Basophil/Eosinophil
Immunologic	T lymphocyte
	B lymphocyte
	NK cells
Growth and differentiation	T lymphocyte
	B lymphocyte
	Epithelial
	Fibroblast
	Bone marrow
Catabolic	Cartilage/Osteoclast
	Synovial
Pyrogenic	Hypothalamus

1.6 IL-1 involvement in disease states

As traumatic, infectious and inflammatory diseases may result in near simultaneous appearance of IL-1, IL-4, IL-6, TNFα, IFNγ and C5a, synergistic and/or inhibitory actions are obviously clinically relevant.

1.6.1 Septic shock syndrome

The clinical syndrome of Gram-negative bacterial septicemia appears to be the result of excessive stimulation of the host system by LPS leading to, amongst other effects, fever, intravascular coagulation and circulatory shock.

The septic shock syndrome is characterised by multiple cytokine release (TNFα, IL-1α and IL-1β, IL-6, IL-8, CSFs, TGFβ) from macrophages and monocytes, as well as release of numerous tissue factors and of antagonists such as IL-1ra. TNFα is thought of as being the central mediator and the mechanism of IL-1 involvement is associated with its ability to increase plasma concentrations of small mediator molecules such as platelet-activating factor, prostaglandins and nitric oxide, all of which are potent vasodilators (Dal Nogare et al., 1991; Dofferhof et al., 1991).

There appears to be differential regulation of IL-1 and TNF during endotoxin tolerance. Injection of LPS into mice elicits transient increase in both factors. TNF appears rapidly followed by IL-1 at 3-4h. Early IL-1 production may be partially controlled by TNF but a second LPS injection at 20h results in a significant increase in serum IL-1 but no further response by TNF (Zuckerman et al., 1991).

IL-6 may again be an important effector of IL-1β and TNFα actions *in vivo*. Fever attributed to LPS induction of IL-1 and TNFα is thought to be mediated by IL-6 (LeMay et al.,1990; Shalaby et al., 1989).

The sepsis syndrome in humans is consistently associated with marked increases in the number of IL-1R expressed on circulating PMN without there being any concommitant increase in complement receptor 3 (Fasano et al., 1991). The mechanism and functional significance is at present unknown but presumably the increase occurs as a result of the increased production of circulating IL-1.

The multiple actions triggered by LPS are detailed in a comprehensive review by Lynn and Golenbock (1992).

1.6.2 Autoimmune related diseases

1.6.2.1 Rheumatic diseases

Several distinct cytokine patterns have been noted in synovial fluid from rheumatic patients. Immune activation pathways appear to be different in rheumatoid (RA) and osteoarthritis (Westacott et al., 1990; Kirkham, 1991). Cells with dendritic morphology and bright IL-1α staining circulate in the blood of RA patients but are not seen in OA or normal subjects. IL-1β is not detected in these cells (Barkley et al., 1990). Serum IL-1β concentrations are higher in RA than in other arthritic conditions, whereas IL-2, TNFα, IFNα, and IFNγ are all lower. No correlation has been found between IL-1β and any other cytokine in synovial fluid. Strong evidence for the involvement of IL-1 in RA is given by an assessment of RA patients with symmetric and asymmetric knee joint inflammation which shows that IL-1β levels in all inflamed joints are significantly higher than in contralateral uninflamed joints (Rooney et al., 1990). Relatively high numbers of dendritic cells are found in RA effusions during acute flares of the disease but it is only macrophages which make the real contribution to the IL-1 detected. IL-6 has been detected in these exudates and implicated in the pathogenesis of RA (Bhardwaj et al., 1988, 1989). IL-1β has been shown to correlate with disease progression and *in situ* work has shown macrophages to be the major source of IL-1β within the rheumatoid synovium (Eastgate et al., 1988; Wood et al., 1992). There is some evidence that B cell derived IL-1α and β, both of which are greatly increased in RA, may be involved in the B cell clonal expansion of RA (Yamamura et al., 1990). The involvement of IL-1α in RA has been difficult to determine (Eastgate et al., 1991) but circulating cells of dendritic morphology which contain IL-1α (but not IL-1β), do appear to correlate with RA but not OA disease activity (Barkley et al., 1990).

Rheumatoid synovial fibroblasts have the ability to secrete large amounts of the matrix-degrading metalloproteinases, collagenase and stromelysin. Two metalloproteinases, the 92K (type V collagenase) and 68K (type IV Collagenase) gelatinases, appear to be differentially and independently regulated by cytokines. Although a large range of cytokines can stimulate collagenase synthesis, only IL-1, TNFα and lymphotoxin can induce the 92K gelatinase. which is constitutively expressed in rheumatoid but not normal cells. These three mediators are involved in potentiating the matrix-degradative phenotype in rheumatoid synovial fibroblasts (Unemori et al., 1991). In a canine model of osteoarthritis, stromelysin, IL-1α and IL-1β were found to be localised in superficial synovial lining cells, infiltrating mononuclear cells, endothelial cells and smooth muscle cells of blood vessles whereas the oncogenes, *c-fos*, *c-jun* and *c-myc*, were detected predominantly in the synovial lining cells (Pelletier et

al., 1993). The latter are thought to be the mediators through which IL-1 induces metalloproteinase expression.

1.6.2.2 Insulin-dependent (type I) Diabetes Mellitus

Activated mononuclear cells appear to be important effector cells in autoimmune pancreatic beta-cell loss leading to insulin-dependent (type 1) diabetes mellitus and there is evidence that systemic IL-1β, but not IL-1α, is directly involved in the specific cell destruction (Bendtzen et al., 1986; Reimers et al., 1991). Recent evidence indicates that IL-1 induced nitric oxide and subsequent inhibition of mitochondrial enzymes could contribute to this destruction (Dimmeler et al., 1993). In mice, type I diabetes has been found to be linked to the IL-1 type I receptor (see Sect. 1.11 for IL-1 receptors) on chromosome 1 (Cornall et al., 1991).

1.6.3 Bacterial meningitis

There is controversy over the involvement of IL-1β in bacterial meningitis and damage to the blood-brain barrier. TNFα appears to be the main culprit, but whether IL-1 synergises with TNF or acts by inducing it has not been clarified. IL-1 does not, however, seem to correlate with any cerebro-spinal fluid indices so far measured (Quagliarello et al., 1991; Sharief et al., 1992).

1.6.4 Renal function

Monocytes produce increased amounts of IL-1β in long term dialysis and end-stage renal failure patients (Schiller et al., 1991). Local production of IL-1, TNF and platelet activating factor in glomeruli by infiltrating or resident inflammatory cells may alter the growth pattern of of glomerular cells and the composition of the secreted matrix leading eventually to glomerulosclerosis (Camussi et al., 1990). IL-1 induces mesangial cell proliferation (Zoja et al., 1991) and has been proposed to play a role in the development of lupus nephritis and immune complex nephritis.

1.6.5 Inflammatory Bowel Disease

Ulcerative colitis and Crohn's disease are characterised by infiltrating activated neutrophils and macrophages which spontaneously release IL-1 (Satsangi et al., 1987). Clinical trials with IL-1ra have been initiated on patients with ulcerative colitis (Dinarello and Wolff, 1993). IL-1 and IL-8, a cytokine with neutrophil-chemoattractant and neutrophil-stimulating properties and which is induced by IL-1, are both high in inflammatory bowel disease but there is also evidence for the presence of regulators of IL-1α and IL-1β induced T-cell activation in Crohn's disease. These regulators, which correlate with disease activity, are not autoantibodies and do not inhibit fibroblast production of PGE_2 (Brynskov et al., 1991).

Paratuberculosis (Johne's disease) is a chronic enteric infection caused by *Mycobacterium paratuberculosis,* most often affecting ruminants. Monocytes from cattle naturally infected with *Mycobacterium tuberculosis* spontaneously release high levels of IL-1. Although the role of cytokines has been examined in related human diseases such as Crohn's disease, only very recently has data on cattle appeared, which suggests that blood monocytes in infected cattle are either non-specifically activated with respect to IL-1 production or possess a defective regulatory mechanism (Kreeger et al., 1991).

1.6.6 Other Disease Implications

Spontaneous release of IL-1 from monocytes and macrophages has been noted in patients with diseases such as sarcoidosis (Hunninghake et al., 1984), tuberculosis (Chensue et al., 1986), scleroderma (Alocer-Varela et al., 1985), and tuberculoid leprosy (Watson et al., 1984). Macrophages and dermal dendritic cells are known to play a central role in the lesions of all these diseases. In lepromatous leprosy, which is characterised by a lack of responsiveness to *Mycobacterium leprae* (Kaplan et al., 1987), blood monocyte-derived macrophages do not produce IL-1 on initial stimulation with *M. leprae,* but can be induced to do so by addition of cyclo-oxygenase inhibitors like indomethacin. This restoration of activity is *M. leprae* specific (Ridel et al., 1986). Reversal reactions, which constitute the type I lepra state and represent naturally occuring delayed-type hypersensitivity reactions favouring macrophage activation and protective immunity, appear to be characterised by increased production of IL-1 (Sarno et al., 1991; Uyemura et al., 1992).

In connective tissue diseases such as systemic lupus erythematosus, Sjogren's syndrome and progressive systemic sclerosis, secretion of both IL-1α and IL-1β is significantly upregulated in monocytes from patients with active disease. The intracellular levels remain normal, there being no correlation between the intra- and extracellular concentrations (Aotsuka et al., 1991).

IL-1 functional activity in psoriatic lesions is reduced, relative to normal skin, this reduction being attributed to increased IL-1ra, reduced IL-1α levels and an IL-1β which lacks activity in T-cell and thymocyte assays. It has been postulated that IL-1β is processed to a novel form which is important in skin homeostasis (Cooper et al., 1990a,b).

IL-1 has been suggested as an autocrine growth factor in myeloid leukemia although some cells do not appear to process IL-1β to its mature form. An inhibitor, probably IL-1ra, is also released by monocytic leukemic cells (Cozzolino et al., 1989). Carcinoma lines elaborating colony-stimulating factors

have shown frequent co-production of IL-1α and IL-6 but not necessarily of IL-1β (Okuno et al., 1991).

1.6.7 IL-1 and Virus Infection

Macrophage infiltration is a constant feature of human viral infections and virus induced production of cytokines would be expected but data on the interaction of IL-1 and viruses is frequently contradictory.

Viruses such as CMV have been reported to reduce or abrogate the ability of thymic epithelial cells and macrophages to produce IL-1 (Wainberg et al., 1988; van Bruggen et al., 1989). The immediate early genes of HCMV are capable of regulating IL-1β expression which may have some influence on the inflamatory processes associated with the infection (Iwamoto et al., 1990). However, a 95K inhibitor, which is not IL-1ra, is also produced from virus-infected monocytes and could contribute to lack of detectable IL-1 activity (Roberts et al., 1985; Rodgers et al., 1986)

Under endotoxin-free conditions HIV has been reported not to induce IL-1, IL-6 or TNF from peripheral blood mononuclear cells. IL-1 may activate HIV via induction of the cellular DNA binding protein NF-κB which activates the HIV LTR by binding to *cis* -acting sequences although TNF reportedly activates HIV more efficiently than IL-1 (Clouse et al., 1989; Kobayashi et al., 1989). Further data shows IL-1 to be capable of inhibiting the anti-viral action of IFNβ, whereas TNF seems rather to exhibit an additive effect with IFNβ (Kohase et al., 1988).

Interestingly, two vaccinia virus surface proteins show homology to human and murine IL-1 and IL-6 receptors. A method of immune evasion by the virus has been postulated in which these virus proteins may bind IL-1 and IL-6 hence preventing interaction of the cytokines and their receptors, consequently decreasing the inflammatory response and increasing virus replication, a mechanism utilised by several pox viruses (Smith et al., 1993).

Infection of monocytes with bovine viral diarrhoea virus results in the production and/or activation of an as yet uncharacterised soluble inhibitor of IL-1 activity which primarily affects thymocytes or immature T cells. IL-1 levels are not affected by the virus (Jensen and Schultz, 1991).

1.7 Cloning and Expression of IL-1

1.7.1 IL-1 Cloning

Initially, cDNAs were isolated by differential screening of a cDNA library from LPS stimulated monocyte polyA$^+$ RNA using probes from unstimulated and stimulated monocyte polyA$^+$ RNA. Use of a goat anti-IL-1 raised against purified murine IL-1 (Mizel et al., 1983) subsequently identified the proteins expressed by *in vitro* translation from the rabbit reticulocyte system (Lomedico et al., 1984; Giri et al., 1985). At the start of the study described in this thesis, IL-1 from human (Auron et al., 1984; Furutani et al., 1985; March et al., 1985; Cameron et al., 1986; Gubler et al., 1986; Tocci et al., 1987), murine (Lomedico et al., 1984; Gray et al., 1986; Huang et al., 1988), rat (Nishida et al., 1988a) rabbit (Furutani et al., 1985; Mori et al., 1988) and bovine (Malisewski et al., 1988) sources had all been cloned and sequenced and the majority expressed as recombinant proteins. The porcine IL-1α sequence was subsequently published (Malisewski et al., 1990). Equine IL-1β has been characterised but not yet cloned (May et al., 1990). IL-1α and IL-1β display marked cross species conservation but are themselves about 40% similar at the nucleic acid level and only about 25% similar at the amino acid level.

The complete IL-1 genomic sequences of the human IL-1α and IL-1β and murine IL-1β genes are known and the organisation of each is very similar (Furutani et al., 1989; Clark et al., 1986; Bensi et al., 1987), see Sect 1.8.

1.7.2 Expression of Recombinant IL-1

E. coli, rabbit reticulocyte lysates, *Xenopus laevis* oocytes, *COS-7* monkey cells, hamster ovarian cells, *Saccharomyces cerevisiae,* have all been used for expression of human, murine and rabbit recombinant IL-1 (Auron et al., 1984; Lomedico et al., 1984; March et al., 1985; Furutani et al., 1985, 1986; Gray et al., 1986; Gubler et al., 1986; Rosenwasser et al., 1986; Jobling et al., 1988; Baldani et al., 1987). Bovine IL-1 has been expressed in *E. coli* (Malisewski et al., 1988). Cloning showed that the C-terminal portions of each IL-1, corresponding to the mature proteins, were sufficient to generate biological activity and that the primary IL-1α translation product is also active. In addition none of the recombinant proteins contain a stretch of hydrophobic amino acids which would normally comprise a secretory signal.

Fuhlbrigge et al (1988) showed that mouse L cells transfected with murine pro-IL-1α c-DNA expressed biologically active pro-IL-1α, which was neither processed to the 17kD mature form nor secreted. The transfected cells also expressed membrane associated biological activity indicating that the pro-IL-1α

can direct expression of membrane associated IL-1 and that cleavage of the pro-molecule is not required for membrane presentation. In contrast, transfected pro-IL-1β c-DNA did not generate biologically active material.

Initial data on the functionally active sites of IL-1β was published in 1987 by Mosley et al. who showed that deletion of the N-terminal beyond position 133 resulted in a complete loss of receptor binding and biological activity. It has been postulated that processing to the mature form, which induces a conformational change, is required for maximal IL-1β activity (Mosley et al., 1987; Black et al., 1988). Whether the recombinant IL-1β proprotein is active may be dependent on the expression system. If expressed in *E. coli*, the IL-1β primary transcript is not active but if expressed in other systems such as *COS-7* cells or by *in vitro* translation, it does show some activity (Rosenwasser et al., 1986; Jobling et al., 1988).

Human IL-1β is the only IL-1β to possess a glycosylation site in the mature protein, all other species have sites in the proprotein N-terminal sequence. Glycosylated recombinant human IL-1β mature protein has been produced from chinsese hamster ovary cells but only by use of fused hybrid secretory sequences. This protein is inactive until deglycosylated (Baldani et al., 1987; Pecceu et al., 1991).

1.8 Genomic organisation

Both human (Todd and Naylor, 1991; Lafage et al., 1989) and murine (D'Eustachio et al., 1987) IL-1α and IL-1β genes have been located on chromosome 2, the two loci being very closely linked. The human IL-1 genes have been mapped to the 2q13->2q21 region and the murine genes are located in the F region of chromosome 2, approximately 4.7 centimorgans distal to beta-2 microglobulin (D'Eustachio et al., 1987; Boultwood et al., 1989).

The genomic structure of human and murine IL-1 genes are known. (Clark et al., 1986; Furutani et al., 1986; Bensi et al., 1987). Human and murine IL-1β genes are almost identical except for the human exon 7 which has an additional 200bp in the 3' untranslated region. The conservation of gene structure suggests a common ancestor ie. both IL-1α and IL-1β genes consist of seven exons and six introns with similar positioning of intron/exon boundaries. Restriction digests of genomic DNA suggest that IL-1α and IL-1β exist as single copies in the bovine (Malisewski et al., 1988), as well as the human genome. The genes differ in regions known to control transcription (Angel et al., 1987; Hurme et al., 1991; Hunninghake et al., 1992). The IL-1α promoter, in contrast to the IL-1β promoter, lacks a CAT box and has a very poor TATA box. Although both

genes contain the consensus sequence found in PMA inducible genes, IL-1α requires an additional signal for activation. NFβA is known to play a selective role in expression of IL-1β but not IL-1α. IL-1, like many other cytokines which control cell growth, contains AU-rich sequences within the 3' untranslated RNA regions which confer extremely short half-lives on the mRNAs and are also important with respect to imposition of translational blockade (Turner et al., 1989; Kruys et al., 1989).

Polymorphisms have been found in all regions of the human IL-1 genes including the promoter regions which may have disease implications (Clark et al., 1986; Bensi et al., 1987; di Giovine et al., 1992; McDowell et al., 1993; Bailly et al., 1993). Data from unrelated individuals has revealed 6 IL-1α alleles within genomic DNA (Todd and Naylor, 1991; Bailly et al., 1993). Three control regions of the IL-1β gene have been shown to be important in transcriptional regulation; −395bp to −132bp enhances expression; −736bp to −395bp is inhibitory and +387bp to +550bp is the first intron-1/exon-2 region reported to mediate positive IL-1β gene expression (Zhang et al., 1991).

1.9 Physical Properties of IL-1

1.9.1 Crystallographic Structure

Crystallographic and nuclear magnetic resonance studies have shown that both mature IL-1α (Graves et al., 1990) and IL-1β (Priestle et al., 1989; Clore et al., 1990, 1991a,b; Veerapandian et al., 1992) are in the form of a trigonal pyramid or tetrahedron with the interior being strongly hydrophobic. 12 antiparallel β strands form a complex of hydrogen bonds with approximate threefold internal symmetry. Mutational data suggests the presence of three distinct binding sites for the IL-1 receptor on the surface of the molecule. It is postulated that each of the three immunological domains which comprise the extracellular portion of the IL-1 receptor recognises one of these sites.

Amino acids known to form the binding site of IL-1β are all situated on the outside of the molecule (Labriola-Tompkins et al., 1991). The putative nuclear location sequence is away from the residues involved in receptor binding and is situated on one of the exterior folds of the IL-1β molecule (Grenfell et al., 1991). Comparison of sequence aligments shows interior residues to be well conserved and exterior residues markedly less so (Priestle et al., 1989). IL-1α shows similar folding patterns (Eriksson et al., 1991; Zhu et al., 1991).

Topologically equivalent folds have also been found in human basic fibroblast growth factor, bFGF, and bovine acidic FGF. These latter two proteins are

members of the FGF family which stimulate proliferation and differentiation of a variety of cell types through receptor-mediated pathways. Although IL-1β and bFGF possess only 10% sequence identity, they represent a family of structurally related mitogenic factors (Zhu et al., 1991; Zhang et al., 1991; Eriksson et al., 1991).

1.9.2 Glycosylation and Phosphorylation of IL-1

Pro-IL-1α is glycosylated and is phosphorylated at Ser-90 which is in the amino terminal third of the precursor. Phosphorylation presumably increases resistance to proteolytic cleavage. (Beuscher et al., 1988; Kobayashi et al., 1990). There is no evidence that natural IL-1β is either glycosylated or phosphorylated.

1.10 Systemic clearance of IL-1

From studies on mice (IL-1β: Newton et al., 1988) and rats (IL-1α: Poole et al., 1990) it has been shown that the major route of clearance is the kidney. Intravenous injection of IL-1β shows a rapid initial loss of IL-1 from the circulation followed by a slower loss over the next hour. Intraperitoneal or subcutaneous injection gives an initial peak in circulating IL-1 after 10min which is sustained over at least the next 7h. Circulating IL-1 is associated with the plasma fraction and is not cell associated. Two hours after injection most tissues contain an equal amount of IL-1 on a weight basis, with the exception of bone which contains half the amount, and kidney which contains 4-8 fold more. 10-20% of the label is found in the urine within hours of injection of IL-1β. The IL-1 found in the urine is intact 17kD IL-1.

IL-1α in rats has a plasma half-life of 2.5min. Unlike IL-1β, 5kD and 9kD fragments of IL-1α are detectable in the urine and intact IL-1α is only found in the plasma. *In vitro* studies on kidney homogenates show that IL-1α is endocytosed and hydrolysed by lysosomal proteinases.

1.11 IL-1 receptors

Two IL-1 receptors, Type I (IL-1RI) and Type II (IL-1RII) have so far been identified on human, murine and porcine cells (Savage et al., 1989; Sims et al., 1988; Chizzonite et al., 1989a,b). IL-1RI and IL-1RII represent different gene products but map to the same chromosomal location (Copeland et al., 1991). Human IL-1RI maps to chromosome 2q12 and IL-1RII to 2q12->2q22, the same region as the human IL-1 genes. In contrast, murine IL-1R genes map to the centromere-proximal region of chromosome 1, a different location to the murine IL-1 genes on chromosome 2. A specific soluble receptor from human monocytes and RAJI B cells has also been characterised (Symons et al., 1991a,b).

1.11.1 Cellular IL-1 Receptors

1.11.1.1 Structure

The molecular weight of IL-1RI is 80-85kD and of IL-1RII, 60-68kD. Both receptors are members of the Ig super family and consist of three segments (Sims et al., 1988; Dower et al., 1990; McMahan et al., 1991); 1) the extracellular segments of approximately 300 residues, which are 28% homologous, each consisting of three Ig-like domains and possessing several potential glycosylation sites; 2) the highly homologous transmembrane segments of roughly 21 residues; 3) non-homologous cytoplasmic domains of about 213 (IL-1RI) and 29 (IL-1RII) residues. The cytoplasmic segment of IL-1RII posses no significant similarity with any sequence in either the GenBank or EMBL databases.

Although there is evidence that N- and O-linked patterns of IL-1RI glycosylation differ with cell lineage, which may affect relative binding of IL-1α and IL-1β (Mancilla et al., 1989) there is also evidence that glycosylation is not essential for binding but may be a prerequisite for subsequent function (Sect. 1.12). Removal of N-linked oligosaccharides leaves a receptor protein core of 50-60K which shows some loss of activity but retains the expected K_d (Urdal et al., 1988).

1.11.1.2 IL-1R Distribution

It was originally thought that IL-1RI was expressed by T lymphocytes, fibroblasts, chondrocytes, hepatocytes, synovial lining cells and epithelial cells and IL-1RII by bone marrow cells, B cell lineages and polymorphonuclear leucocytes but there is accumulating evidence that the segregated distribution to different cell lineages may not be so straightforward.

In addition to the predominant 80K type I receptor, 40K, 60K, 75K, 100K and 220K molecular weight receptors have also been detected on murine T cell lines by cross-linking of IL-1 (Bird et al., 1987; Chizzonite et al., 1989; Savage et al., 1989). It has been suggested by some that the Type I receptor on EL4 cells is a heterodimer consisting of the 40/80K proteins (Kroggel et al., 1988) and by others eg. EL4 and NOB-1 and Balb/C 3T3, is an 80/102K doublet (Solari et al., 1990a,b). Deglycosylation reduces the smaller receptor by a further 12K. Glycosylation patterns may again be affecting signal transduction from the different receptor molecules.

Human B cells only express the 60K form and murine B cells a 75K form of receptor (Solari et al., 1990a,b). Some B cell lines, however, exhibit a heterogeneous population of receptors, expressing 68K and 80K or 110K forms,

the two larger of which bind IL-1α with a greater affinity than IL-1β (Benjamin et al., 1990; Cronkhite et al., 1993). It has been suggested that the 110K receptor is a dimer. Some EBV B cell lines express an 80K receptor (Benjamin et al., 1990) and one report suggests that monocytes may also express an 80K receptor (Uhl et al., 1989).

These differences are becoming resolved by data such as that of McMahan et al (1991) who have shown that several different cell lines, including B lymphoblastoid lines, HepG2 cells and peripheral blood T cells, can be induced to express mRNA and protein for both receptor types, presumably in order to utilise different signal transduction pathways. They have also indicated preliminary, and as yet unpublished, evidence that the two receptors cannot bind IL-1 simultaneously.

1.11.1.3 Binding Properties of the IL-1 Receptors

Bird and Saklatvala, in 1986, detailed initial studies which showed that natural porcine IL-1α and IL-1β both bound to IL-1 receptors on porcine, human and murine connective tissue cells in a specific fashion and with similar affinities. Chondrocytes displayed 7,000 sites per cell with K_d 2.5 x 10^{-10}, fibroblasts and osteoblasts had 3-5,000 sites with K_d 1.5 x10^{-10}. In numerous subsequent studies in various species, recombinant IL-1α and IL-1β have been found to bind to both receptors but with affinities dependent on receptor type (eg. Dower et al., 1986; Horuk et al., 1987; Ghiara et al., 1989; Savage et al., 1989). The only exception so far described, is the central nervous system in which specific IL-1β receptors which do not bind IL-1α are heterogeneously distributed in the brain (Katsura et al., 1988).

Scapigliati et al first demonstrated differential binding of IL-1α and IL-1β to receptors on T and B cells. Murine thymoma EL4-6.1 cells, predominant receptor 80K Type I, express more receptors per cell for IL-1α (22656, K_d 3.6 x 10^{-10}) than for IL-1β (2988, K_d 1 x 10^{-9}) but IL-1α and IL-1β are mutually competitive. Human B lymphoma RAJI cells, predominant receptor 68K Type II, were shown to have a higher number of binding sites for IL-1β (2400, K_d 1.3 x 10^{-10}) than for IL-1α (316, K_d 2.2 x 10^{-9}). IL-1α could effectively be displaced by both IL-1α and IL-1β while only IL-1β could displace bound IL-1β. IL-1 binding to the Type I receptor is rapid and followed by internalisation of the complex whereas type II receptor binding is slower and the complex does not appear to be internalised efficiently (Chizzonite et al., 1989; Benjamin et al., 1990; Bomsztyk et al., 1989; Ghiara et al., 1989; Horuk et al., 1987; Scapigliati et al., 1989). One documented exception is of rapid rhIL-1α binding to a PMN receptor of 60-70K, with subsequent rapid internalisation at 37°C (Rhyne et al.,

1988). These IL-1RI and IL-1RII binding patterns hold true in general for human and murine cells. IL-1 receptor binding data for numerous cells types is given in detail in Appendix A.1. In general, untransformed cells or cells isolated from tissues or blood express few IL-1 receptors, ie. 100-500 per cell, but some cell lines express extremely high receptor numbers, ie. $>10^4$.

High and low affinity classes of receptor have been detected on a large number of cell types. The functional significance of the measured affinities is unclear as cells with the type I receptor can respond to femtomolar concentrations of IL-1, far below reported dissociation constants for even the high affinity class. It has also been suggested that some T cells can respond to subpicomolar concentrations of IL-1 without demonstrable binding (Rosoff, 1990). This may be consistent with the observation that less than 5% receptor occupancy can trigger phopshorylation of the remaining receptors (Gallis et al., 1989). It has been proposed that as few as 1-10 molecules per cell can elicit biological responses and that these responses are mediated through the lower rather than the higher affinity IL-1R (Dower et al., 1986; Qwarnstrom et al., 1988).

From the small number of studies which report binding of IL-1 at a single cell level, it appears that only a proportion of either resting cells, $\leq$16%, or activated cells, $\leq$30%, bind IL-1 (Tanaka et al., 1989; Stoppaciaro et al., 1991; Chin et al., 1987).

1.11.1.4 Internalisation of IL-1

IL-1 bound to the IL-1RII is poorly internalised, remains bound to the surface for up to 60 minutes and is found in the extracellular fluid in a degraded form (Horuk and McCubrey, 1989; Horuk, 1991; Shelly et al., 1992) whereas IL-1 bound to the IL-1RI is internalised within minutes via an azide sensitive mechanism and can remain inside the cell for 12 hours (Matsushima et al., 1986; Uhl et al., 1989; Horuk et al., 1987; Horuk, 1991). Most of the internalised IL-1 is undegraded, retains binding activity and accumulates both on the nuclei in a receptor specific fashion and in lysosomes. The putative IL-1β nuclear localisation sequence is a seven amino acid sequence (-PKKKMEK-; hβ-208-214) which shows some identity with the nuclear localisation sequence of SV40 large T-antigen (Roberts, 1989). The structurally similar bFGF, contains the same peptide sequence in a similar position and also accumulates at the nucleus after internalisation (Grenfell et al., 1989).

This sequence is well conserved across all IL-1βs so far cloned, including bovine giving reason to believe that it will also be conserved in ovine IL-1β. Such conservation strongly indicates functional significance. Grenfell et al (1989) demonstrated that IL-1 binding to EL4 nuclei is specific and saturable

and shows similar kinetics to the plasma membrane receptor. Several lines of evidence from IL-1 internalisation profiles using fibroblasts and EL4 cells, also suggest that receptor-mediated endocytosis of IL-1 and transport to the nucleus may be required for generation of a biological response and that nuclear translocation may occur with IL-1 still complexed to its receptor (Mizel et al., 1987; Qwarnstrom et al., 1988; Falk et al., 1989; Curtis et al., 1990). On the other hand it has also been claimed that biological potency of IL-1β is not necessarily related to its internalisation and nuclear accumulation (Grenfell et al., 1991; Heguy et al., 1991). The suggestion that nuclear transport may not play a significant role in IL-1 signal transduction is consistent with the fact that IL-1 bound to truncated mutants of the IL-1RI extracellular domains can be internalised without initiating a full signal transduction cascade (Dower et al., 1989). Hisactophilin, a unique actin binding protein of similar 3-D structure to IL-1β and bFGF but with an unrelated primary amino acid sequence, triggers signal transduction but only at pH values below seven (Habazetti et al., 1992) which may have some relevance to IL-1 elicited signals. Signalling through IL-1/IL-1R binding is discussed further below (Sect. 1.12).

The IL-1α proprotein contains a polybasic sequence (-LKKRRL-; hα-81-86) which might serve as a nuclear localisation signal but the mature IL-1α has no obvious signal.. Nuclear accumulation of mature IL-1α has been detected in murine thymoma EL4 6.1 cells and specific saturable binding to isolated nuclei has been demonstrated (Grenfell et al., 1989). The IL-1 type I receptor has a potential nuclear localisation signal in its cytoplasmic tail (-VKKSRR-) (Sims et al., 1988) which may serve to target membrane-receptor-bound IL-1α. More recently, Weitzman and Savage (1992) have reported nuclear internalisation of IL-1α in numerous cell lines and demonstrated DNA binding by both IL-1R and the IL-1α/IL-1R complex, but not by IL-1α alone.

1.11.1.5 Regulation of IL-1R Expression

Specific and different stimuli appear to be required for upregulation of each receptor. IL-1RI expression can be upregulated by other cytokines eg. IL-3, IL-4 and GM-CSF (Dubois et al., 1989; Lacey and Erdmann, 1990), growth factors eg. fgf and PDGF (Chandrasekhar et al., 1989; Bonin and Singh, 1988;) and PGE_2 or cAMP generating agents eg. forskolin (Akahoshi et al., 1988a; Takii et al., 1992). IL-1RII is upregulated by DEX and glucocorticoids (Scapigliati et al., 1989; Stoppciaro et al., 1991; Akahoshi et al., 1988a). DEX can induce IL-1RI as well as IL-1RII mRNA in monocytes but only the latter appears to be translated (Spiggs et al., 1990). IL-1 can upregulate both IL-1RI and IL-1RII (Akahoshi et al., 1988b; Takii et al., 1992; Shieh et al., 1990; McMahan et al., 1991; Dubois et al., 1991). PMA drastically reduces IL-1RII expression but has

very little effect on IL-1RI expression (Bomstzyk et al., 1989; Lowenthal and MacDonald, 1986) despite having an equally potent effect in activating PKC.

Upregulation increases both the number of cells expressing receptors and the number of receptors expressed per cell. Binding affinity is not generally altered by upregulation but some human B lymphoblastoid lines do exhibit decreased affinity with increased receptor expression (Stoppacciaro et al., 1991). Induction of IL-1 receptors seems to require synthesis of both new RNA and protein, actinomycin D and cycloheximide both inhibit the induction of type II receptors by GC (Akahoshi et al., 1988a,b). DEX can induce IL-1RI mRNA without inducing surface expression of the receptor.

The half-life of the type II receptor on B cells is 2 hours, shorter than that of the type I receptor on T cells, 5-12 hours (Horuk et al., 1989).

1.11.1.6 The IL-1/IL-1R Interaction

Antibody studies indicate that the binding domains of the IL-1RI for the two IL-1 species are distinct (Ikejima et al., 1990), contributing to the different biological responses to IL-1α or IL-1β which can be effected from the type I receptor. It is postulated that all three immunological domains which comprise the extracellular portion of the IL-1 receptor are involved in generating an active IL-1 binding-site (Dower et al., 1989, Bomstzyk et al., 1989b, Clore et al., 1991b). Minimal data is available on IL-1RII binding.

The structure/function relationships of IL-1 receptor binding still require much clarification but various IL-1 residues have so far been implicated as being essential either for receptor binding or for generation of biological responses. Structural specifications appear to be less rigid than those determining biological activity. The residues detailed below are numbered with respect to the human sequences unless otherwise indicated.

The N-terminal regions of the IL-1 mature proteins show greatest conservation and several reports have indicated that alteration of amino acids in this region affects IL-1 activity without altering binding affinities ie. residues α-134–155 and β-126–144.

For IL-1α activity (Yanofsky et al., 1990; Nanduri et al., 1991; Poindexter et al., 1991; Kawashima et al., 1992), there appears to be an absolute requirement for a basic α-128 residue. Other important residues are α-Leu-136, α-Asp-138, α-Glu-142, α-Ile-144, α-Leu-152. Apart from two conservative differences in the murine sequence, residues 136-143 are conserved across all IL-1αs and 141-143 constitute a potential receptor binding site. Although some of these residues are

conserved in IL-1β, β-Asp-128 is the only equivalent residue so far to have been implicated in activity. α-Asp-263 and its equivalent β-Asp-261 are the only important C-terminal residues to have been noted. α–Asp-138 and α–Asp-263 which appear to be essential for lymphocyte activation, cytostatic activity against human melanoma cells and PGE_2 induction are fully conserved across IL-1α and IL-1β of all species and are spatially situated very close to one another. They may therefore constitute one of the active sites for IL-1/IL-1R binding (Kawashima et al., 1992). Further evidence for this comes from mutation of Lys to Arg at the equivalent position in the IL-1 receptor antagonist (see Sect 1.13.II), which results in the antagonist displaying minimal IL-1-like activity (Ju et al., 1991).

Seven residues, Arg-120, Leu-122, Phe-162, Ile-172, Lys-209, Lys-219 and Glu-221, have been proposed to be the major contributors to a discontinuous IL-1β/IL-1RI binding site (Chang et al., 1992; Nanduri et al., 1991; Veerapandian et al., 1992; Yem et al., 1992). There is absolute conservation of all seven residues apart from bovine β-Gln-117, β-Ile-119, β-Arg-206, which align with the human β-Arg-120, β-Leu-122, β-Lys-209 respectively and represent conservative changes. The 3-D structure of IL-1β shows that these essential residues are all clustered in one region (Labriola-Tompkins et al., 1991). β-Asn-224 which has only a minor effect on binding is on one edge of the site and five residues found to be non-essential for binding lie on the periphery.

Several pieces of evidence indicate that IL-1β activities can be dissociated, for example, anti-IL-1β studies have shown that residues β-148–192 are not implicated in the binding to IL-1RI, despite containing two of the residues supposedly involved in the discontinuous binding site, but are important for T cell activation (Boraschi et al., 1991b; Herzbeck et al., 1989). The peptide 161-173, which lies in the hinge region between β-strands 4 and 5 (Priestle et al., 1989) is immunostimulatory but does not induce the majority of inflammation-related IL-1β activities (Tagliabue et al., 1991). (See Sect 1.14 for adjuvant activity of this peptide). The epitope for immunostimulation may be different to that for stimulation of bone resorption (Lerner et al., 1991) and some peptide sequences have been shown to reproduce only a limited range of IL-1 activities, eg. hyperalgesia (Ferreira et al., 1988) and somnogenesis (Obal et al., 1990).

Point mutations at different sites can uncouple IL-1 activities, for example, mutation of β-Arg-120 results in markedly reduced pyrogenic potency while retaining normal immunostimulatory activity and ability to induce hematopoetic growth factors (Nanduri et al., 1991; Nakai et al., 1990). Mutation of Gly for β-Arg-127 in the human or murine sequence has no effect on receptor binding but

causes failure of T cell proliferation (Gehrke et al., 1990; Conca et al., 1991). Residues in the bovine sequence equivalent to human β-Asp-120 and β-Arg-127 are both Glu, indicating possible species differences in binding requirements, an idea highlighted by the lack of reactivity of bovine IL-1β on murine cells (Malisewski et al., 1988). Similarly deamidation of the unique murine β-Asn-149 causes loss of receptor binding and co-mitogenic activity (Daumy et al., 1991) but the equivalent human and bovine residues, Glu and Lys respectively, which may indicate a degree of species specificity.

The putative nuclear location sequence is away from the residues involved in receptor binding and is situated on one of the exterior folds of the IL-1β molecule, thus making it accessible for nuclear membrane binding, whether or not IL-1 is bound to its receptor at this stage. According to Mosley et al. (1978b), the IL-1RI does not bind proIL-1β but this data has not been corroborated by any other groups.

Data on IL-1RII requirements is lacking but residues β-166-169 and β-177-186 have been implicated as binding peptides (Boraschi et al., 1991a). βPhe-162 is the only one of the residues of the IL-1RI discontinuous binding site which has any effect on IL-1RII binding. βAsp-261 is essential for IL-1RII binding (Ju et al., 1991).

Substitution of either Met-160 or Val-174, results in the loss of binding to both types of receptor. These residues are fully conserved across IL-1α and IL-1β and the side chains containing them are both buried, confirming their probable importance for the structural stability of IL-1.

Patterns of IL-1α and IL-1β cross-linking to the doublet receptor found on EL4 cells are not identical (Solari et al., 1990a,b). Murine Th1 cells (MTg12B), which are involved in delayed type hypersensitivity reactions, express the 60K form of receptor but do not respond to IL-1 even though it does bind to the cells. Th2 cells express both 80K and 60K forms but only selected functions are affected ie. IL-1 promotes cell growth, but does not enhance the constitutive IL-4 production by these cells (Solari et al., 1990b, Williams and Unanue, 1991).]

IL-1α does not compete efficiently with IL-1β for type II binding which indicates different binding requirements whereas competition for type I binding indicates similar IL-1α and IL-1β requirements. The mutational and antibody data described above indicates that the contact points in each receptor/ligand complex are probably different and determined by the 3-D structures and different residues may be involved in the binding to the two different types of receptor.

1.11.2 Soluble IL-1β Receptor (sIL-1R)

A soluble 47K receptor from the human B cell line, RAJI, specifically binds human or murine IL-1β, both proprotein and mature protein, which is not displaceable by either IL-1α or by IL-1 receptor antagonist (Symons et al., 1991a,b). This soluble receptor demonstrates a similar affinity to that of the RAJI cell surface receptor, ie. 2200pM, and appears to be a proteolytically cleaved form of this Type II receptor. Cells possessing a Type II receptor which binds IL-1α and IL-1β equally eg. U937, do not release the sIL-1R suggesting either structural heterogeneity within IL-1RII, lack of a specific protease or a third type of receptor. The same authors have also reported a specific IL-1β binding protein from PHA stimulated human monocytes which has the same properties. LPS or IL-1α stimulation does not consistently induce synthesis of this binding protein.

1.12 IL-1 receptor signalling (IL-1 signal transduction)

Elucidating the events which occur once IL-1 has bound to cells has been a controversial area and there is still no consensus of opinion. Cytokines such as IL-1 bind to cells and induce various reactions including ultimately, the transcriptional activation of target genes and a cascade of biological reactions. At the different levels of activation the signals are known as first messengers (extracellular signals), second messengers (initial intracellular signals such as cAMP and kinases) and third messengers (transcription factors). Transcription factors mediate transcriptional induction by interacting with specific elements in the regulatory regions of genes hence influencing their expression. Cytokines are thus in turn themselves regulated by transcription factors.

1.12.1 IL-1 as a First Messenger

The two IL-1 receptors appear to employ different signal transduction pathways. From studies on the human IL-1 receptor antagonist, signaling through the type I receptor is the dominant pathway for IL-1 induction of transient neutrophilia, elevation of hepatic acute phase proteins and movement of PMN from bone marrow to local sites of accumulation (McIntyre et al., 1991) but may not be required for antigen-specific cell-mediated or humoral responses (Faherty et al., 1992). It has been postulated by Stoppacciaro (1991) that the type II receptor is involved in initiation of the cell cycle because of the responsiveness of cells in G_0-G_1 phase to IL-1. There is also evidence that IL-1 can drive the cycling of bone marrow cells (Neta et al., 1987) as well as initiate their differentiation (Mochizuki et al., 1987), presumably by stimulation of specific growth and differentiation factors.

The cytosolic region of IL-1RI has no homology with any known protein kinase but the serine/threonine residues are phosphorylated soon after IL-1 has bound to the extracellular domains (Gallis et al., 1989). The complex is internalised (Qwarnstrom et al., 1988; Curtis et al., 1990) and the cells are triggered to respond. Heguy et al (1991) have presented evidence that IL-1 internalisation and nuclear localisation are not sufficient to trigger IL-1 activation of gene expression in T-cells. The cytoplasmic domain of the receptor is not involved in the internalisation process but a 50-amino acid segment in the cytoplasmic domain of the fibroblast IL-1R has been shown to be critical for signal transduction. This region is strikingly conserved across human, murine and chicken IL-1Rs. A functional homology between IL-1R and the Drosophila toll protein has been suggested (Heguy et al., 1992). The fate of IL-1 bound to the Type II receptor is less clearly defined although the shortened IL-1RII cytoplasmic domain may explain some of the differences in signal transduction from the two receptors.

A number of mechanisms possibly involved in IL-1 signal transducion have so far been elucidated but there is still much debate as to exactly how IL-1 exerts its influence and it appears that no single signal transduction pathway is sufficient to explain all the effects of IL-1 on its many target cells.

1.12.2 Second Messengers and Protein Kinases

Most cell activators lead to a change in the levels of specific second messengers such as diacylglycerol (DAG) or cAMP, activators of protein kinases C and A (PKC, PKA) respectively. Subsequent phosphorylation of discrete substrates ultimately leads to the cellular response.

Reports from several laboratories indicate that IL-1 can activate serine/threonine kinases. Tyrosine-specific phosphorylation does not seem to be common (Lovett et al., 1988) but tyrosine kinases are rapidly recruited in the early stages of signalling (Guy et al., 1991). Data on PKC and/or PKA involvement in IL-1 induced protein phosphorylation is contradictory but the evidence does not point to either being obligatory.

Effects of IL-1 can certainly be mimicked by activators of PKC such as phorbol myristyl acetate (PMA) eg. stimulation of collagenase and PGE_2 production (Postlethwaite et al., 1983; Taylor et al., 1988) but many IL-1 induced phosphorylations such as of serine residues in the cytosolic P65 protein and the EGF receptor as well as serine and threonine residues of a triad of small heat shock protein hsp 27 isoforms, are PKC independent (Shiroo et al., 1990; Kaur et al., 1989; Bird and Saklatvala, 1990). P65 phosphorylation is also not attributable to PKA or calmodulin-dependent kinases.

Changes in DAG or cAMP levels have not been consistently detected although both classes of receptor have been shown to be capable of inducing increases. The phospholipid source for increased DAG seems to vary with cell type ie. hydrolysis of phosphatidylethanolamine in mesangial cells, phosphatidylinositol in macrophages and phosphatidylcholine in T cells. The latter at least, appears to be PKC independent (Mills et al., 1989). Murine macrophage IL-1R is possibly activated by rapid, 5s, hydrolysis of phosphoinositides and generation of the second messenger, inositol trisphosphate (IP3) (Wijelath et al., 1988). Although activation of adenyl cyclase does not seem to be an obligatory feature of IL-1 signalling, occupancy of Type I IL-1R has been shown to activate a tyrosine kinase and generate the accumulation of intracellular cAMP but in amounts considered not great enough to stimulate phosphorylation. Neither IL-1 nor TNF increase cAMP levels in monocytes, Type IIR (Shiroo and Matsushima, 1990) and lack of significant effects of IL-1 on cAMP metabolism in cells expressing either type of receptor has also been demonstrated by RayK et al. (1992).

Further evidence for lack of PKC/PKA involvement is given by IL-1 being able to stimulate rapid phosphorylation of its own receptor on serine/threonine residues despite the absence of demonstrable protein kinase activity intrinsic to, or associated with the receptor (Gallis et al., 1989). In addition there are indications that PKC is not translocated from the cytosol to the membrane (Shiroo and Matsushima, 1990). On the other hand, IL-1 appears to be capable of activating PKC and PKA through independent IL-1R in the same T cell line (Munoz et al., 1991,1992b). Whether this variability arises from different types of receptor expression or plasiticity in response coupling of IL-1R, as suggested by Farrar et al. (1990) has not been determined although Chedid et al. (1991) have proposed that the level of AP-1 activity in T cells may be determined by the balance between several serine/threonine protein kinases and phosphatases.

Two proposals with respect to elevation of cAMP have been put forward, i) that it may be secondary to induction of prostaglandins ie. via increased arachidonic acid and cyclooxygenase (Bomsztyk et al., 1990; Oppenheim et al., 1980) and ii) that the IL-1R is coupled to a G1-like guanine nucleotide binding protein, G protein which activates adenyl cyclase (Chedid et al., 1989; O'Neill et al., 1990). Studies involving the bacterial pertussis toxin, have indicated a role for G protein, in IL-1 signal transduction, in fibroblasts and in the membrane of the IL-1 receptor-rich strain (NOB-1) of the murine EL4 thymoma line (O'Neill et al., 1990;; Rollins et al., 1991; Chedid et al., 1989). IL-1α is ten-fold more potent than IL-1β and acts by increasing the affinity but not the number of GTP binding sites. GTPase activity in the membrane in also increased in a dose dependent

manner (O'Neill et al., 1990). It has also been postulated that IL-1R function may be regulated by guanine nucleotides via a higher affinity state for IL-1 binding, a mechanism different to that exhibited by conventional G-protein-linked receptors. Evidence that IL-1β induction of cGMP and nitric oxide is blocked by the protein synthesis inhibitor cycloheximide, implicates both of these in IL-1 effects (Corbett et al., 1992).

In the light of such contradictory data, a role for PKC/PKA in IL-1 signalling remains controversial. Some kinase other than these two had been thought to be involved in the signal transduction, and evidence for sphingomyelin signalling pathway activation by IL-1β in both dermal fibroblasts and EL4 thymoma cells has recently been presented (Ballou et al., 1992; Mathias et al., 1993). This signalling pathway, which is also utilised by TNFα, involves hydrolysis of sphingomyelin to ceramide and stimulation of a ceramide-activated Ser/Thr kinase. Another potential candidate is mitogen activated protein 1 (MAP-1), a serine/threonine kinase (Bird et al., 1992).

1.12.3 Induction of Transcription Factors by IL-1

Changes in transcription are mediated by DNA binding proteins, many of which are activated by phosphorylation. Of these, AP-1 and NF-κB are of particular interest with regard to IL-1 and have been detected in a number of IL-1 responsive genes. AP-1 is the transcription factor with major involvement following stimulation of the cell membrane and is the first IL-1 responsive element. NF-κB serves to enhance transcription from cellular genes and also mediates interactions between host cells and viruses.

IL-1 induces the active nuclear form of the DNA binding protein, NF-κB, in both T and B cells but otherwise the activation pathways seem to be different in the two cell types. In T cells and fibroblasts the activation is via AP-1 elements whereas in B cells the NF-κB activation involves cAMP.

The proliferative response of T cells to mitogens or cytokines involves the transcriptional induction of several cellular genes, prominent among these being the immediate early nuclear proto-oncogenes *c-fos* and *c-jun* and the late *c-myc*, c-myb and N-ras which encode transregulatory proteins important in the control of gene expression (Curran and Franza, 1988). *c-fos* and *c-jun* form a stable heteroduplex, the AP-1 transcription factor, which binds to the AP-1 binding site present in the 5' upstream region of many cellular genes such as those of metallothionine IIA, collagenase, IL-2, the cis-control region of SV40 virus and a region of the HIV LTR. The affinity of the *fos/jun* heterodimer is much greater

than that of a *c-jun* homodimer and *c-fos*, being unable to homodimerise, does not bind DNA. (Munoz et al., 1992a). From mutational studies with IL-1β, *c-fos* and *c-jun* expression is not necessarily accompanied by increased transcription of genes containing the AP-1 binding site and it seems probable that at least two events are required for IL-1-mediated late gene induction in fibroblasts. Both IL-1β and a mutated R/G127 form can stimulate transcription of *c-fos, c-jun,* and the early IL-1β and IL-6 genes, but the mutein can not induce transcription of the late procollagenase and prostromelysin genes (Conca et al., 1991).

IL-1 has been shown to induce *c-fos* and *c-jun* genes in T helper type 2 (Th2) cells via different signal transmission pathways (Munoz et al., 1990a). *c-jun* gene transcription appears to depend entirely on a tyrosine kinase pathway which is independent of PKC and cAMP production and which is linked to the 80K IL-1R. *c-fos*, on the other hand, seems to be absolutely dependent on, and positively regulated by, the PKC pathway. In one particular T-cell line, LBRM, which possesses a large number of IL-1 receptors, *c-fos* could be activated by mitogens whereas *c-jun* was exclusively induced by IL-1 in the same cells 10 - 15 minutes later (Munoz et al., 1992). *c-jun* complexes with the glucocorticoid receptor whereas *c-fos* can form complexes with other proteins and can act through sequences other than AP-1 (Gurney et al., 1992). Phorbol esters and calcium ionophores also induce c-jun, hence mimicking of IL-1 action (Mantovani and Dejana, 1989).

Of the other proto-oncogenes, *c-myc* is significantly induced by IL-1 in fibroblasts through positive regulation of NF-κB (Kessler et al., 1992).

The conventional view of NF-κB is that the unactivated form is held as an extranuclear complex with an inhibitor IκB. Phosphorylation of IkB by protein kinases including PKA and PKC, causes dissociation of the complex and the liberated NF-kB translocates to the nucleus (Ghosh and Baltimore, 1990). This pathway could operate in the IL-1 mediated activation of NF-κB in EL4, 70z/3 and YT cells (Chedid et al., 1990) but data from rat PC60 cells rather suggests that IL-1 activates NF-κB molecules already present in the nucleus (Espel et al., 1990).

Other IL-1-activated DNA-binding proteins exist, the best characterised so far being NF-IL-6, which binds to a 14-bp sequence element in the IL-6 promoter, which shares homology with a liver-enriched nuclear factor C/EBP and has been shown to be essential for IL-1 inducibility (Akira et al., 1990). A motif within the a_1-acid glycoprotein promoter has been reported to respond to nuclear factors produced by IL-1 in fibroblasts and hepatocytes (Prowse and Bauman, 1988).

1.13 IL-1 inhibitors and binding proteins

1.13.1 IL-1 receptor antagonist (IL-1ra or IL-1 receptor antagonist protein, IRAP) Early studies described the presence of an inhibitor in the supernatant of human monocytes cultured on adherent immune complexes which had potent effects on IL-1 stimulation of thymocytes, chondrocytes, fibroblasts and synovial cells (Arend et al., 1985, 1989). Now characterised, it is known as IL-1 receptor antagonist (IL-1ra), a specific antagonist produced predominantly by monocyte/macrophages but also by polymorphonuclear leukocytes and keratinocytes. What appears to be an identical inhibitor has been found in the urine of patients with monocytic leukemia (Seckinger et al., 1987) and also in supernatants from PMA induced U937 cells (Carter et al., 1990). Human IL-1ra has been mapped to chromosome 2, in the same location as other members of the IL-1 family, but contrary to IL-1, the IL-1ra mRNA does not contain the AUUUA sequence implicated in regulating the IL-1 mRNA half-lives (Eisenberg et al., 1990).

IL-1ra is a 152-residue, 22-25K glycoprotein with a pI of 5.0 which, contrary to IL-1, does have a signal peptide. The amino acid sequence shows 41% homology with IL-1β and 30% with IL-1α. Although IL-1ra is structurally related to IL-1β, it specifically blocks both IL-1α and IL-1β binding to the receptors without itself activating the target cells (Eisenberg et al., 1990; Carter et al., 1990; Arend et al., 1990). Murine IL-1ra appears to exhibit IL-1R binding competition only with IL-1α (Matsushima et al., 1991). The IL-1 type I receptor binds IL-1ra selectively, but not exclusively and the unglycosylated 17K recombinant IL-1ra as expressed by *E. coli* is equally efficient at blocking IL-1 binding. The type II receptor has been shown to bind IL-1ra but with a much lesser affinity (Granowitz et al., 1991). IL-1ra probably plays an extremely important role in in controlling the immunoregulatory balance during immunity and inflammatory processes. It follows that the type I IL-1 receptor represents the predominant signaling pathway for many host responses attributable to IL-1. Specific antagonists to IL-1RII, once found, will aid elucidation of the exact function of this receptor.

The recently cloned human IL-1ra promoter has been found to be selectively active in cell lines in which the endogenous gene is active (Smith et al., 1993). Experiments at both molecular and protein levels (Arend et al., 1991b; Poutsiaka et al., 1991; Turner et al., 1991; Vannier et al., 1992) have established that IL-1 and IL-1ra production by monocytes are differentially regulated by mediators including LPS, TGFβ and IL-4. Recent reports suggest that moncytes constitutively produce IL-1ra during maturation into macrophages, an effect enhanced by GM-CSF (Roux-Lombard et al., 1989; Janson et al., 1991). Again

it is suggested that the balance between the production of IL-1 and its antagonist may be important for the regulation of the immune response and chronic inflammation during pathological processes. As an example, the activity of IL-1 increases and of IL-1ra decreases with respect to normal, in cells from healthy smokers and patients with interstitial lung diseases (Takeuchi et al., 1992). Very recently it was suggested that the induction of IL-1ra by GM-CSF and IL-4 occur via different mechanisms as GM-CSF can induce IL-1β co-ordinately in U937 cells. In these cells IL-1ra expression is not affected by IL-2, IL-3, IL-5, IL-6, IFNγ, M-SCF, G-CSF, TNFα, growth factors or IL-1ra itself (Berger et al., 1993).

IL-1ra regulates IL-2-related functions by a suppressive action on the T lymphocyte (Kashiwado et al., 1989). In this context, studies on AIDS patients, which are at best confusing with regard to whether IL-1 production is normal or impaired, suggest increased synthesis of IL-1ra with the balance of IL-1/IL-1ra production favouring receptor inhibition. As T cell function in these patients is impaired together with a lessening of factors which augment IL-1 synthesis but not IL-1ra, it has been suggested that excessive production of IL-1 inhibitors may contribute to immunodeficiency (Berman et al., 1987; Chensue et al., 1992).

Keratinocytes have been found to produce constitutively large amounts of a biologically active intracellular variant of IL-1ra (icIL-1ra) which is 20K in size, unglycosylated, lacks a leader sequence and has an additional seven N-terminal amino acids. Both forms of IL-1ra are derived from the same gene through use of an alternative transcriptional start site and internal splice-acceptor site (Haskill et al., 1991). Epidermal IL-1ra is found in both forms (Hammerberg et al., 1992). With differentiation of the keratinocytes, the IL-1ra/IL-1 ratio increases, showing increased IL-1ra mRNA (Bigler et al., 1992; Gruaz-Chatellard et al., 1991). This is also true for both forms of IL-1ra in psoriatic skin (Hammerberg et al., 1992). Non-secreted IL-1ra may therefore serve to regulate autocrine IL-1-mediated pathways of growth/differentiation. In addition, glucocorticoid treatment of epidermal cells abolishes IL-1 but not IL-1ra production indicating that immunosuppressive effects of glucocorticoids, as well as inflammatory effects of IL-1α in human skin, are modified by IL-1ra (Stosic-Grujicic and Lukic, 1993).

Examples of the beneficial effect of IL-1 blockade are that IL-1ra protects against IL-1 induced arthritis in rabbits (Henderson et al., 1991), has also been shown to protect adrenalectomised rats against the lethal effect of IL-1

(Mengozzi et al., 1991) and prevents death due to LPS administration in rabbits (Ohlsson et al., 1990).

Reducing the pathogenic role of IL-1 by blocking its actions is a potential strategy for treating patients with acute and chronic diseases. IL-1ra has been administered to normal subjects in a phase I trial, which showed that even at plasma concentrations of 25-30μg/ml, no abnormal diagnostic indices were detectable (Granowitz et al., 1992). This is consistent with non-involvement of IL-1 in normal homeostasis. Clinical trials involving short term infusion of IL-1ra have shown potential benefit and trials involving patients with ulcerative colitis have been initiated (Dinarello, 1993). Future trials will have to determine to what extent blocking the action of IL-1 action may impair host defences during prolonged treatment.

1.13.2 Other Inhibitors and IL-1 Binding Proteins

Uromodulin, an immunosuppressive glycoprotein found in the urine of pregnant women, binds to IL-1α and IL-1β but also to TNF and other cytokines and is therefore non-specific (Hession et al., 1987; Brown et al., 1991).

Naturally occuring cytokine binding proteins include lipoproteins, lipids, acidic polysaccharides and alpha-2 macroglobulin (α2M). α2M is a major plasma binder whose native and activated forms bind different cytokines selectively. IL-1β is bound preferentially by trypsin- or methylamine-reacted, ie. activated forms of α2M through a thiol ester-dependent mechanism. α2M probably acts by regulating the distribution, clearance and bioactivity of circulating IL-1 (Borth and Luger, 1989; LaMarre et al., 1991; Kaplan and Nielsen, 1979). Even co-valently bound IL-1β is biologically active and labelling experiments have established that the binding is specific, via Cys-122, and can only be inhibited by IL-1 (Borth et al., 1990; Teodorescu et al., 1991).

Several other IL-1 inhibitors have been described. A specific 52K IL-1 inhibitor is produced by the human myelomonocytic M20 cell line and by the mouse macrophage P388D cell line (Barak et al., 1991; Isono and Kumagai, 1989) and virus infection can induce release of two additional inhibitors of 8K and 95K from PMN and Mϕ (Berman et al., 19; Roberts et al., 1986; Rodgers et al., 1985). UV irradiated or hydrocortisone treated keratinocytes produce two basic (pI 8.8) inhibitors of 40 and 50K (Schwarz et al., 1987; Kupper and McGuire, 1986). An IL-1 inhibitor, of 95K molecular weight is produced by virus-infected monocytes *in vivo* and *in vitro* and contributes to the lack of detectable IL-1 activity (Rodgers et al., 1985; Roberts et al., 1986). None of the above appear to be forms of the specific IL-1 receptor antagonist and the relationship, if any, between these various inhibitors has not been established.

IL-1R-related binding proteins have been detected. IL-1 is considered to be a major link in the neuroimmune axis and shared saturable one way transport of IL-1α and IL-1β from blood to brain has been demonstrated. The blood-brain transporter appears to be similar, but not identical to, the T lymphocyte (TypeI) receptor and has greater affinity for IL-1α than IL-1β (Banks et al., 1991). The soluble IL-1β specific binding protein (see 1.11.2) is generated by proteolytic cleavage of the extracellular portion of the type II receptor.

1.13.3 IL-1 Autoantibodies

Autoantibodies to IL-1α and IL-1β have been detected by several groups in normal human plasma as well as in patients in a number of disease states, some of which are quoted as examples.

Data on the prevalence of IL-1 autoantibodies is inconsistent because of differing methods of detection but trends are discernable from within individual studies. From receptor binding studies, 25% of normal plasmas tested contained anti-IL-1α and 2% contained anti-IL-1β IgG antibodies (Gallay et al., 1991). The binding capacity and affinity were found to be greater in male than female sera (Svenson et al., 1990).

Patients with rheumatoid arthritis have been reported to have neutralising IgG anti-IL-1α autoantibodies; 16% of RA patients compared to 6% of normal subjects and 5% of patients with systemic lupus erythematosis. Antibody titres, as assessed by RIA, were shown to fluctuate with RA disease activity (Suzuki et al., 1990, 1991). Anti-IL-1α autoantibodies have also been seen at a higher frequency in sera from patients with Schnitzler's syndrome (Urticaria, bone pain, fever, macroglobulinemia). The predominant class was IgG but in some patients IgA was also detected (Saurat et al., 1991).

These antibodies have potent neutralising capacity and a high receptor binding capacity and are thus distinct from other cytokine autoantibodies. The presence of these high affinity IL-1α autoantibodies even in the sera of normal individuals, has prompted the suggestion that they could act as specific physiological carriers and regulators (Bendtzen et al., 1990). Receptor binding and IL-1α epitope studies are required to help elucidate their pathological significance.

1.14 IL-1β as an adjuvant:

For quite some time, IL-1β has been mooted as an effective adjuvant for poorly immunogenic vaccines, for example IL-1β and lipoxygenase metabolites have been shown to mediate the lethal effect of Freund's adjuvant in adrenalectomised rats (Peretti et al., 1991). IL-1β with point mutations which yield proteins

retaining immunostimulatory activity but with greatly reduced pyrogenicity are all adjuvant candidates. The synthetic IL-1β 163-171 peptide is extremely effective as an adjuvant, being completely devoid of inflammatory or toxic effects and has been recommended for human use (Tagliabue et al., 1991). Hybrid proteins in which foreign sequences have been inserted into IL-1β without changing the overall structure have also proven useful for delivery of therapeutic agents (Wolfson et al., 1991).

IL-1α does not exhibit adjuvant properties but rather acts as a competitive inhibitor of IL-1β adjuvanticity (Boraschi et al., 1990).

1.15 IL-1 and Antigen Presentation

1.15.1 T Cells

Freshly derived human $CD4^+$ T cells are divided into naive and memory T cells based on the expression of the reciprocal CD45 isoforms, CD45RO and CD45RA respectively (Swain et al., 1991). The requirements for IL-1 in the activation of naive and memory T cells appear to be different. IL-1, including membrane IL-1α, plays a critical role in the early phases of a primary response but it seems to have little effect on the later phases or on the responses of memory T cells (Plebanski et al., 1992). Human prestimulated $CD4^+$ T helper cells, but not $CD8^+$ cells, acquire the ability to stimulate monocytes to produce IL-1. Cell to cell contact is a prerequisite for this stimulation *in vitro* (Dunlap and Tilden, 1991). An earlier study showed that CD8+/CD4- cytotoxic T lymphocytes have secrete IL-2 once the IL-1R is triggered by IL-1 after antigen stimulation (Klarnet et al., 1989).

Murine studies show IL-1 to be required for optimal clonal expansion of memory cells in response to stimulus and differential expression of the IL-1 receptor mRNA correlates with their responsiveness to IL-1 (Luqman et al., 1992). Accessory cells can significantly regulate T helper cell (Th) effector function at the level of cytokine production (Bloom et al., 1992; Williams and Unanue, 1991). The Th1 subset, involved in delayed hypersensitivity, produces IFNγ and IL-2 and does not require IL-1 for proliferation. The Th2 murine $CD4^+$ subset activate B cells and produce IL-4. They express both forms of IL-1 receptor (80 and 60K) and whilst IL-1, especially IL-1α in the membrane bound form, is the requisite costimulator for the growth of Th2 murine T-cell clones (eg. D10.G4.1 cells used for IL-1 bioassay), IL-4 production is not enhanced. Antigen presentation reversibly upregulates both forms (80 and 60K) but stimulation with Concanavalin A only upregulates the 60K form (Solari et al., 1990b; Williams and Unanue, 1991).

Human B cells, which are also involved in antigen presentation, only express a few IL-1 receptors until stimulated with *Staphylococcus aureus* Cowan I (SAC) ie. after stimulation the major low affinity class, increased from 320 to 1960 per cell, K_d 3.8 x 10^{-10} and the minor high affinity class increased from 70 to 300 per cell, K_d 4.4 x 10^{-12} (Tanaka et al., 1989). Anti-class II antibodies also inhibited the number of receptors on these cells indicating that class II may play a part in the activation of these receptors.

1.15.2 Dendritic Cells

Dendritic cells are a system of antigen presenting cells which function to initiate several immune responses such as the sensitisation of MHC-restricted T cells, the rejection of organ transplants, the formation of T-dependent antibodies and presentation of antigens in lymph nodes. DC, cells with a dendritic, veiled, cell morphology which are rich in surface class II histocompatibility antigens (Ia antigens) but lacking in other lymphocyte or monocyte markers, are found in many non-lymphoid tissues but can migrate via the afferent lymph or the blood to the T-dependent areas of lymphoid organs. They constitute a small proportion (<1%) of circulating mononuclear cells. In inflammatory joint effusions they constitute up to 7% (Tsai et al., 1989) and in peripheral afferent lymph, in which the cells of dendritic morphology are derived mainly from Langerhans cells in the local skin area, they constitute 5-10% of the monocyte population (Hopkins et al., 1989). Follicular dendritic cells, although morphologically similar, are thought to be functionally distinct and have not come under consideration in the current study. Table 1.2 is a resume of DC distribution. Also included is the potential for IL-1 production (± indicates conflicting evidence).

TABLE 1.2

Tissue Distribution of Cells with Dendritic Characteristics and Associated IL-1 Production

Tissue	Dendritic Cell Type	Characteristics	IL-1 Production
Epidermal	Langerhans' cell	Antigen uptake/processing	±
Afferent lymph	Veiled	Antigen transfer	±
Lymph node	Interdigitating	Antigen processing	?IL-1β
Spleen	"	"	–
Tonsil	"	"	–
Peripheral blood	Peripheral blood	Migratory form of DC	±
Thymic medulla	Thymic T	Tolerance induction	?
Thymic reticulum	Thymic R	?Antigen presentation	IL-1α
Parenchymal organs	Interstitial	?Antigen uptake/processing	?
Germinal centres	Follicular	Regulation of B cell function	?

DC occur in two states of differentiation. In the immature state they are highly specialised to process foreign protein antigens and in the mature state they efficiently stimulate resting antigen-specific T cells. DC can migrate from the non-lymphoid tissues, where they reside in the immature state, via the afferent lymphatics or the blood to the T cell dependent areas of the lymphoid organs (lymph nodes, spleen), where they appear as mature cells.

Following exposure to sensitising chemicals, dendritic cells rapidly accumulate in the draining lymph nodes. A proportion, at least, of the DC arriving at the nodes bear significant amounts of antigen and are derived from LC. Although LC are relatively inefficient antigen-presenting cells, the antigen-bearing DC found within the draining node are potent accessory cells and induce immune responses both *in vivo* and *in vitro*. As LC migrate from skin to lymph node they are subject to a phenotypic maturation and as they develop into DC they acquire active antigen-presenting function (Macatonia et al., 1987). It has been shown that after skin painting mice on one flank, there is an increase in DC in the collateral and distal lymph nodes in addition to the draining nodes but only DC in the draining nodes carry antigen (Hill et al., 1992). Peripheral lymph node DC, irrespective of whether they are derived from resting or draining lymph nodes and irrespective of whether they are bearing antigen show elevated membrane Ia antigen expression (Cumberbatch et al., 1991).

DC are generally thought to be of hemopoetic stem cell origin but if so, it is not known at what stage of monocyte development the priming for development into LC/DC occurs but autocrine stimulation may play a part (Reid et al., 1990; Rossi et al., 1992a). There is histological evidence from heterologous skin graft experiments that cutaneous regions can also serve as sources of LC (Demarchez et al., 1993).

DC rapidly upregulate adhesion molecules *in vitro* (Teunissen et al., 1990) which is important with respect to the immunogenicity of antigen containing DC. Recent studies have shown that stratum corneum-derived human IL-1 injected into the skin will upregulate dermal dendritic cell expression of the adhesion molecules ICAM-1 and VCAM-1 (Groves et al., 1992).

During two or three days of culture epidermal Langerhans cells (LC) develop from immature into mature cells. GM-CSF is the major stimulus for maturation of DC (Caux et al., 1992; Heufler et al., 1988) and IL-1 and TNFα have both been strongly implicated as initiators of the migration of antigen-containing LC to lymph nodes (Cumberbatch and Kimber, 1992, Vakkila et al., 1990). There is

evidence that both interdigitating thymic reticulum cell and LC/DC function may be dependent on IL-1α but not IL-1β or TNF production (Ruco et al., 1990).

Veiled cells in afferent lymph were first described in 1978 (Kelly et al.) and have become the subject of increasing interest as potent antigen presenting cells. It is not known exactly how the cells take up antigen and although early reports indicated a lack of phagocytic activity and antigen processing this now appears to be incorrect. The presence or absence of Fc receptors on DC has been variously reported. Murine lymph node and spleen (Nussenweig et al., 1981) and human peripheral blood DC (van Voorhis et al., 1982) do not appear to express Fc whereas rat lymph node (Schalke et al., 1985), murine pulmonary tissue (Sertl et al., 1986) and Langerhans cells (Spry et al., 1980) and human afferent lymph dendritic cells do (Witmer-Pack et al., 1988). Both Fc positive and negative DC have been found in sheep afferent lymph (Bujdoso et al., 1990). It is possible that varying states of differentiation are being seen. FcR negative cells have been reported as having the greatest antigen presenting capacity (Inaba et al., 1988). However, Harkiss et al (1989) have shown that approximately two thirds of sheep afferent lymph DC bind antigen/antibody complexes via Fc receptors.

Some *in vitro* studies indicate that DC process proteins for a short period of time while the rate of synthesis of MHC products and content of acidic endocytic vesicles are high (Pure et al., 1990). Flow cytometry evidence reveals that DC are metabolically active and that traffic through the late endosome does occur in a pattern of fluid-phase endocytosis similar to that described for other antigen presenting cell types (Levine and Chain, 1992). Recently nitric oxide, which is synthesised from arginine as part of a major route by which activated cells of the macrophage lineage mediate cytostasis of the cells, has been demonstrated in rat thoracic afferent lymph DC following IFNγ exposure, although the NO detected in lymph DC was markedly less than from macrophages (Denham and Barfoot, 1992).

Antigen presentation by dendritic cells requires antigen/Ia (class II) complex for specific recognition of antigen. There is evidence that DC may direct local, as opposed to systemic, immunity by controlling T cell cytokine production (Spalding et al., 1983, 1986; Everson et al., 1992). Data on the interaction of cytokines and DC from various sources is beginning to appear, much of it contradictory and very little is yet known about IL-1 and the LC/afferent lymph DC/Lymph node axis. Human tonsil DC do not produce IL-1α or β and neither is IL-1 essential for DC-stimulated allogeneic T lymphocyte responses (Calder et al., 1992). The same authors have been unable to demonstrate IL-1R or TNFαR

mRNA by PCR, in peripheral blood DC. IL-1 mRNA or IL-1 activity has been found in DC by some but not all groups (Koide et al., 1987b, 1988; Vakkila et al., 1990; Waalen et al., 1986). DC from inflammatory sites have been found to produce large amounts of IL-1 spontaneously whereas DC from peripheral blood produce less IL-1 than autologous monocytes (Waalen et al., 1986).

DC maturing from LC in culture have been found to produce large amounts of IL-1β (Ruppert and Peters, 1991). However, SJ Hopkins et al (1990) have shown that IL-1 production *in vivo* is a very early event and that levels of IL-1 in lymph fluid after contact sensitisation are very low. Circumstantial evidence points to IL-1 being produced by the DC in the lymph but this remains to be clarified.

An amplifying rather than an initiating role is currently being ascribed to IL-1. From much of the published work on DC as antigen presenting cells, it appears that IL-1 is not essential as a second signal for allogeneic T-cell responses, that IL-1α, IL-1β and IL-6 are primarily monocyte derived and are neither required nor produced during the activation of resting T cells by DC and that proliferation is not blocked even by high concentrations of anti-IL-1 antibody (Bhardwaj et al., 1989; McKenzie et al., 1989; Vakkila et al., 1990; Naito et al., 1989). GM-CSF is a major macrophage activating factor which, apart from inducing cytokines, also induces macrophage HLA-DR expression (Chantry et al., 1990). There are a number of reports of direct augmentation of the DC/Tcell reaction by GM-CSF and/or IL-1 (Koide et al., 1988; Wilson et al., 1988). Rabbit DC activity is enhanced indirectly by IL-1 containing supernatants (Kapsenberg et al., 1985) as is murine DC function, which has been shown to be enhanced if DC are exposed to IL-1 prior to addition to an MLR assay (Koide et al., 1987a). The enhancement is thought to be via direct action on the DC, not the T cell but the mechanism whereby IL-1 enhances DC function has not yet been elucidated.

IL-1 seems to be produced during monocyte but not dendritic cell mediated T cell proliferation (Koide et al., 1988). In primary antigen-dependent systems like the MLR, IL-1 production is not detected in accessory (DC or Mϕ) or T cells, but monocytes can be induced to make IL-1 after interaction with sensitised antigen specific T cells. Both alloreactive T cell clones and freshly prepared lymphoblasts can induce IL-1 provided the monocytes carry the HLA-DR antigens to which the T cells were initially sensitised. It seems that cell contact rather than an IL-1 inducing factor leads to IL-1 production. Neither DC nor B cells seem to make much IL-1 even under these conditions (Bhardwaj et al., 1989).

1.16 Ovine Afferent Lymph

Because of the ease of cannulation of its lymphatic ducts, the sheep is ideal for the study of cells in afferent lymph. Using this system, fresh cells can be continuously collected hence obviating cell culture, which could potentially affect the characteristics displayed by the cells. Cannulation also allows detection of early *in vivo* events following antigen challenge of the draining skin area. Much work has been done in our department on the characterisation of sheep afferent lymph dendritic cells (Bujdoso et al., 1989, 1990; Hopkins et al., 1989; Harkiss et al., 1990). The majority of DC express surface immunoglobulin (Ig) of IgM and IgG1 types. At least four subpopulations of dendritic cell enter the lymph node via the afferent lymph - CD1 positive and negative, Fc receptor positive and negative. It is not yet clear whether these represent distinct cell types or cells in a transitional state. Class II positive, Fc positive DC are known to be efficient at presenting antigen to $CD4^+$ T cells. Whether there is restriction amongst the lymph DC subsets with respect to antigen presentation has not been clarified, but the CD1 positive, Fc negative cell has been proposed as the principal presenting cell for γδ T cells via a heat shock protein/CD1 ligand which would obviate the necessity for Fc. Sheep afferent DC constitutively express $\pm$ 3 x 10^5 class II molecules per cell but cells entering the lymph node from a site of secondary antigen challenge express a sixfold higher level. CD4, LFA-3 and its ligand CD2, which are involved in signal transduction and intermolecular binding, are all expressed as DC cell surface markers. Human studies have shown that perturbation of LFA-3 causes IL-1 release (Le et al., 1987) but it is not itself upregulated by IL-1α or LPS (Swerlick et al., 1991).

The adhesion molecule ICAM-1 (CD54) is upregulated by IL-1α (Swerlick et al., 1991). A further adhesion molecule, ELAM-1 is only very transiently expressed upon stimulation by IL-1α (Beekhuizen et al., 1991). Interestingly, although IL-1 does not affect adhesiveness of lymph node endothelial cells, synergism of IL-1 with suboptimal doses of IL-4 and TNFα does increase their adhesiveness (Chin et al., 1991).

The proportions of various lymphocyte subpopulations in sheep blood differ markedly from those in blood of other species. The $CD4^+$ and $CD8^+$ subsets are sparse, being 20% and 12% respectively whereas in human blood these subsets are 50-60% and 10-20%. sIg^+ cells are 30% in sheep compared to 10% in human blood. $CD3^+$, $CD4^-CD8^-$ cells comprise 3% of human PBL whereas in sheep, the analogous population, $T19^+$, is present at 15-20%. Because of the different phenotypes in blood and lymph, cells in afferent lymph migrating from

blood in peripheral tissues must presumably be selected at the blood vessel endothelium and it appears that activated cells preferentially enter afferent lymph (Mackay et al., 1990).

1.17 Summary of reasons for the study of ovine IL-1

Although a large amount of data on ovine and bovine leukocyte molecules has been available for quite some time not much has been known about their cytokine interactions. Partly because of the cloning of various bovine and ovine interleukins, data on ruminant cytokine interactions is now accumulating at a reasonably rate.

Two species with IL-1-like activity and characteristics were originally detected from sheep peripheral blood mononuclear cells stimulated with Con A (Harkiss et al., 1989). They induced both cartilage resorption and thymocyte activation but not lysis of the murine L929 cell line which is responsive to TNF. Bovine monocyte-derived IL-1 was first purified by Lederer in 1989.

It is clear that IL-1 plays pivotal a role in the regulation of normal tissue function, the immune response and the pathogenesis of disease. In order to study the involvement of IL-1 in disease states in sheep it is essential to acquire knowledge of the normal reactions to immune stimulation on which subsequent analysis can be based. When this study was initiated it was known that bovine and ovine material were likely to be very similar. Bovine IL-1 c-DNA sequences had just been published but no recombinant protein was available. Species barriers do exist and other less closely related IL-1s might not be able to produce data relevant in the sheep. The aims therefore were to clone and express ovine IL-1 in order to be able to study it's involvement in the immune response. Alveolar macrophages, rather than PBMCs, were used as a source of IL-1 mRNA because of the numbers of cells obtainable and ease of purification.

Cannulated of sheep lymph nodes provide a unique model of the *in vivo* immune response. Afferent lymph drains from the surrounding skin area which can be locally challenged with antigen. When this study was initiated, dendritic cells were known to be extremely important antigen presenting cells but nothing was known about the interaction of IL-1 and the Langerhans' cell derived afferent lymph dendritic cells. Recombinant ovine IL-1, once available, could be used to

determine whether these dendritic cells expressed IL-1 receptors and whether this expression could be upregulated by antigen challenge. By determining the type of IL-1 receptor, DC lineage could be established. The presence of IL-1 mRNA in these cells could be determined at a later stage by *in situ* hybridisation with cDNA probes. This information is of potential importance with respect to the processing of antigen and possible antibody targeting in future disease therapy in the sheep.

CHAPTER 2

MATERIALS AND METHODS

Methods are detailed as they were most frequently used. Specific modifications are noted in the main text where appropriate. Where considered relevant, comments on the methods are included. The methods are ordered, as far as possible, to relate chronologically to subsequent chapters. All tissue culture media were purchased from Gibco and chemicals from Sigma Ltd. unless otherwise stated.

2.1 Animals used in this study

2.1.1 Sheep

Finnish Landrace x Dorset, Grey Faced x Suffolk and Dorset x Suffolk sheep of various ages were obtained from the Moredun Research Institute, Edinburgh.

2.1.2 Rabbits

Dutch albino rabbits from our own breeding colony.

2.2 Reagents and techniques frequently used in tissue culture and nucleic acid work

2.2.1 Tissue culture

2.2.1.1 Media

a. Sterile RPMI 40, Iscoves serum free medium and Dulbecco's MEM, were all supplemented with 5mM glutamine for use. Benzylpenicillin and streptomycin were routinely added at 100 units/ml. Foetal calf serum was added to media as required.
b. Hanks balanced salt solution (HBSS).
c. Phosphate buffered saline (PBS): 137mM NaCl; 26.8mM KCl; 14.7mM KH_2PO_4; 81mM Na_2HPO_4.
d. PBA: PBS containing 0.1% (w/v) bovine serum albumin fraction V and 0.1% Na azide.

2.2.1.2 Incubation conditions for tissue culture

All incubations were at 37°C in a humidified atmosphere of 5% CO_2/95% air.

2.2.2 Nucleic acid manipulations

2.2.2.1 Commonly used reagents

a. 20 x SSC: 3M NaCl; 0.3M Na citrate; pH7.0.
b. TAE: 40mM Tris base; 0.114% glacial acetic acid; 1mM EDTA.
c. TBE: 90mM Tris base; 90mM boric acid; 1mM EDTA.
d. TE: 10mM TrisHCl; 1mM EDTA.
e. TNE: 10mM TrisHCl; 100mM NaCl; 1mM EDTA; pH 8.0.
f. Salmon sperm DNA and yeast RNA: phenol extracted and resuspended at 1mg/ml in water.
g. Klenow fragment of *E. coli* polymerase I: referred to as 'Klenow'.
h. *Taq* polymerase: referred to as 'Taq'.

2.2.2.2 Purification of nucleic acids by phenol extraction and ethanol precipitation

For RNA extraction, redistilled phenol was equilibrated with 0.3M Na acetate pH5.2. For DNA extraction, redistilled phenol was equilibrated with 10mM TrisHCl pH8.0. 0.1% w/v hydroxyquinoline was added to scavenge oxidation products. Redistilled phenol was stored at -20°C for up to a few months and buffered phenol solutions at 4°C for 3-4 weeks.

a. Standard phenol extraction:

Nucleic acid samples were sequentially extracted by vigorous shaking with (i) an equal volume of phenol; (ii) an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v), twice; (iii) an equal volume of chloroform:isoamyl alcohol (24:1 v/v), twice. After each extraction step the solution was centrifuged and the aqueous layer retained. RNA and DNA were finally precipitated by addition of 0.25M Na acetate pH5.2 and 2.5 volumes nucleic acid grade ethanol, either on dry ice for 1 hour or at -20°C for 16-20h. The precipitate was microfuged at 4°C for 15min, washed with 70% ethanol and resuspended in water. RNA samples were stored at -70°C and DNA samples at -20°C.

Unless otherwise indicated, use of the term 'phenol extraction' will encompass the above procedure in full.

b. Hot phenol extraction:

Extraction of DNA from low gelling temperature (lgt) agarose gels included additional initial extraction steps. Lgt was melted at 65°C, extracted first with phenol/chloroform/isoamyl alcohol at 65°C then buffered phenol at 65°C. Subsequent extraction steps followed the normal protocol.

2.2.2.3 Purification of cDNA by absorption to silica

(Geneclean, Bio 101 Inc., La Jolla, California)

The manufacturers instructions for isolation of pure DNA by absorption to silica powder were followed exactly.

2.2.2.4 Measurement of nucleic acid concentration

Nucleic acid concentrations were measured spectrophotometrically.
In solution, 50mg DNA = 1 unit OD_{260nm} and 40mg RNA = 1 unit OD_{260nm}.
The $OD_{260/280}$ ratio at pH7.0 - pH8.0 should be 1.8 - 2.0. If outside this range, a further chloroform extraction was performed.

2.2.2.5 Nucleic acid markers for agarose gels

a. RNA low molecular weight ladder (BRL Inc.): Fragment sizes are 1770, 1520, 1280, 780, 530, 400, 280 and 160 kilobases.

b. RNA high molecular weight ladder (BRL Inc.): Fragment sizes are 9500, 7500, 4400, 2400, 1400 and 240 kilobases.

c. DNA markers: Digestion of λDNA with EcoRI and HindIII restriction nucleases produces fragments of generally suitable sizes, ie. 21226, 5146, 4973, 4268, 3530, 2027, 1904, 1709, 1375, 947, 831, 564, 125 base pairs.

2.3 Preparation of RNA and cDNA

2.3.1 Preparation of alveolar macrophages

Ovine alveolar macrophages were obtained either by lung washout with 200ml HBSS under anaesthetic or by bronchoalveolar lavage with 4 x 500ml HBSS at postmortem. Cells were harvested by centrifugation at 1500 x g for 15min at 4°C, washed twice with 100ml HBSS and resuspended in 9ml HBSS. To lyse any erythrocytes present, 13.5ml H_2O was added, the tube inverted rapidly twice and 1.5ml 10 x PBS added immediately to restore isotonicity. Macrophages were washed and resuspended in 20ml Iscoves serum free medium. Cell concentrations were adjusted as required.

2.3.2 LPS stimulation of alveolar macrophages

Macrophages were allowed to adhere to plastic culture bottles (Nunculon, Denmark) at 37°C for 24h in a humidified atmosphere of 5% CO_2/95% air, prior to stimulation with 10μg/ml or 100μg/ml LPS in fresh medium. Cells were stimulated for various time periods as required, with the 0 hour time point being taken at the time of LPS addition. For experiments in which total RNA was required, a T75 bottle containing 2 x 10^7 macrophages was used for each time

point. If polyA$^+$ was to be prepared, 2 x 10^8 macrophages in T125 bottles were used. Supernatants were stored in aliquots at -70°C and RNA was extracted from the cells.

2.3.3 Preparation of Total RNA from alveolar macrophages

2.3.3.1 Isolation of RNA by centrifugation through CsCl

Culture medium was removed from the adherent macrophages. RNA was extracted from the cells following the method of Chirgwin et al., 1979. Cells were solubilised by addition of 8ml GTC buffer (5M Guanidine isothiocyanate; 25mM Na citrate; 0.5% Na lauryl sarcosinate; 100mM β-Mercaptoethanol; 1mM EDTA) with agitation of the bottle. Solubilised macrophages in GTC can be stored at this stage, either at 4°C for a few days or at -20°C for longer periods. Solubilised cells in a total volume of 11ml GTC were layered over 1.6ml 5.7M CsCl and centrifuged at 76,000g at 20°C for 16h. The supernatant, including the interface band which consists of DNA, was discarded and the tube drained upside down. The sides of tube were dried without disturbing the pellet. The pellet was resuspended in 300ml RNA uptake buffer (0.3M Na acetate pH5.2; 0.1% SDS; 10mM EDTA) by gentle pipetting. RNA was phenol extracted and precipitated with 2.5 volumes cold analar ethanol. Precipitated RNA was pelleted by microcentrifugation, washed with 70% ethanol, resuspended in H_2O and the concentration measured (see Sect. 2.2.2.3).

2.3.3.2 Single-step RNA preparation (Chomczynski and Sacchi, 1987)

2.5ml of Solution D (4M GTC; 25mM Na citrate pH 7.0; 0.5% Sarcosyl) was added to dissolve cells in 75ml culture flask then transferred to a polypropylene tube. Sequentially, 0.1ml 2M Na acetate pH4, 1.0ml water saturated phenol and 0.2ml chloroform/isoamyl alcohol (49:1) were added and the mix shaken vigorously for 10sec prior to cooling on ice for 15min. After centrifugation at 10,000g for 20min at 4°C the aqueous phase was mixed with 1ml isopropanol and left at -20°C for at least 1hr to precipitate the RNA. Proteins and DNA in the interface and the phenol phase were discarded. The RNA was re-precipitated by adding 0.3ml Solution D and 1 vol isoamyl alcohol. The resulting pellet was washed with 75% ethanol, resuspended in 0.5% SDS (as an RNase inhibitor), and stored at -20°C.

2.3.4 Preparation of PolyA$^+$ RNA

PolyA$^+$ enriched RNA was prepared by a modification of the method of Manniatis (1982). All plastic was autoclaved, all glassware was siliconised and baked and all solutions treated with 0.01% diethylpyrocarbonate (DEPC) at 4°C to inactivate RNases. DEPC was removed either by autoclaving or by heating to 68°C for 4h in the case of solutions which could not be autoclaved.

500mg (≤2ml wet vol) oligo dT-cellulose Type7 (Pharmacia) was equilibrated in Eppendorf tubes, by three washes in 0.1M NaOH; 5mM EDTA followed by neutralisation with H_2O and washing with 5 volumes of loading buffer (20mM TrisHCl pH7.6; 0.5M NaCl; 1mM EDTA; 0.1 %SDS). 1mg RNA in as small a volume as possible plus an equal volume of 2 x load buffer was heated to 65°C for 5min, allowed to cool and added to the cellulose in the tube. After 10min the tube was centrifuged, the supernatant reheated, cooled and added to the cellulose. After 5min the supernatant was removed and the cellulose washed with four volumes of load buffer then four volumes of this solution plus an additional 0.1M NaCl. RNA was eluted with 1.2ml buffer (10mM TrisHCl pH 7.5; 1mM EDTA; 0.05% SDS) for 2h at 4°C. Supernatants were concentrated with isobutanol. The polyA$^+$ RNA was precipitated with ethanol containing 0.3M Na acetate pH 5.2 and resuspended in 50µl H_2O.

2.4 cDNA and PCR

2.4.1 cDNA Synthesis

First strand cDNA was synthesised either from total RNA or from polyA$^+$ enriched RNA using Amersham cDNA synthesis kits and either oligo-dT_{12-18} or random hexanucleotides as primer. PCR primer B5.1 (see Figure 3.4) was used to prime the synthesis of cDNA for use in terminal transferase reactions (see Sect. 2.3.5). Parallel labelled and unlabelled reactions were carried out, exactly as per the manufacturers instructions, in order to assess the amount of cDNA synthesised.

2.4.2 Preparation of oligo-dA tailed cDNA (Wensink et al. 1974)

B5.1 primed single strand cDNA was tailed with dATP using the terminal transferase reaction. 110ng cDNA (~2pM) was incubated at 37°C for 90min with 20µM dATP and 10units terminal transferase in cacodylate buffer (100mM NaCacodylate pH 7.0; 1mM $CoCl_2$; 100µM DTT; 50µg/ml BSA) and the reaction stopped by heating to 75°C for 10min. Ideally 10 dA residues should be added per 30min incubation with 10units of enzyme and 4pM DNA hydroxyl ends. Tail length was estimated from autoradiographic exposure of electrophored samples (see Sect. 2.5). Tailed cDNA was used as a template for anchored PCR.

2.4.3 λgt10 cDNA libraries

cDNA libraries were constructed using the Amersham λgt10 cDNA cloning system kit. The manufacturers directions for cloning, screening and isolating cDNA clones were followed exactly.

2.4.4 Polymerase chain reactions

100µl PCR reactions were carried out in 0.5ml Eppendorf tubes with overlaid sterile mineral oil to prevent evaporation. In addition to the cDNA template, reactions contained 10-20pM primers, 100µg/ml BSA, 200µM each of dATP, dCTP, dGTP and dTTP; 2.5units *Taq* polymerase and 10µl buffer. Two buffers were routinely used, Anglian (Anglian Biosystems Ltd) and Ohara (Ohara et al., 1989) see below. Reactions were carried out in a Techne thermal cycler. 35 amplification cycles each consisting of denaturation 93°C/30sec, primer annealing t°C/30sec, polymerisation 72°C/2.5min, were followed by 72°C/5min to allow full extension of synthesised strands. The annealing temperature, **t**, was determined for each primer pair. PCR products were analysed by agarose gel electrophoresis (see **2.3.8.2**).

Anglian PCR buffer: 6.7mM $MgCl_2$; 10mM β-Mercaptoethanol; 6.7µM EDTA; 16.6mM $(NH_4)_2SO_4$; 67mM TrisHCl pH8.8

Ohara PCR buffer: 1.5mM $MgCl_2$; 3mM dithiothreitol; 50mM KCl; 10mM TrisHCl pH8.8

2.5 Detection of Nucleic acids

2.5.1 Electrophoretic separation of nucleic acids on agarose gels

2.5.1.1 Denaturing RNA gel electrophoresis

3 volumes of denaturing solution (60% deionised formamide; 7.2% formaldehyde; 0.1mM EDTA; 20mM phosphate pH 7.0) was added to 10µg RNA (at >1µg/µl in H_2O). After heating to 52°C for 15min, sample dye (30% ficoll; 0.05% Bromophenol blue) and was added at 1:5(v:v) and the sample immediately loaded onto a 1.5% denaturing agarose gel (1.5% agarose; 20mM phosphate pH 7.0; 6.7% Formaldehyde). Gels were run at 40V in 20mM phosphate pH 7.0 for 16h at room temperature with buffer recirculation.

2.5.1.2 DNA gel electrophoresis

DNA samples in load buffer (0.25% bromophenol blue; 0.25% xylene cyanol; 40% sucrose) were electrophoresed through 0.8 – 1.5% agarose 6 x 8cm mini-gels made with TAE/EtBr (0.04M Tris acetate; 1mM EDTA; 0.5µg/ml ethidium bromide). Gels were run in TAE and DNA bands visualised on a UV transilluminator.

2.5.2 Detection of immobilised nucleic acids by hybridisation with radiolabelled cDNA probes

2.5.2.1 cDNA probe labelling (modified from Feinberg and Vogelstein, 1984)

Oligo labelling buffer (OLB) was prepared from the following components.

Solution O consisted of 1.25M TrisHCl; 0.125M $MgCl_2$.

Solution A consisted of 1ml solution O; 18ml β-mercaptoethanol; 5µl dATP, 5µl dGTP, 5µl dTTP (each of which had been previously dissolved in 3mM TrisHCl pH7.0; 0.2mM EDTA).

Solution B consisted of 2M Hepes titrated to pH6.6 with 4M NaOH.

Solution C consisted of Hexadeoxyribonucleotides (P-L No. 2166), evenly suspended in TE at 90 OD units/ml.

Solutions A:B:C were mixed in a ratio of 100:250:150 and stored at -20°C. OLB is not affected by repeated freezing and thawing cycles.

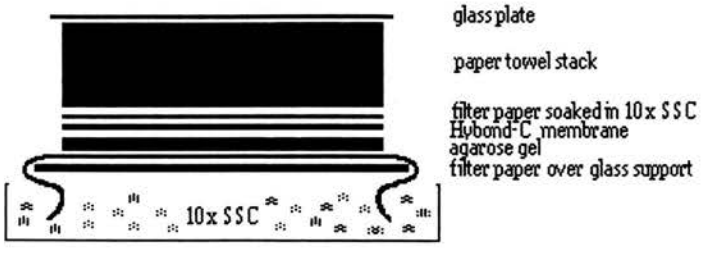
~30ng DNA was boiled for 3min with 6µl OLB in a total volume of 25µl then allowed to cool to room temperature. 40µCi α^{32}PdCTP (10µCi/µl) and 1µl (1 unit) 'Klenow' were added and incubated for 2h at 37°C. ^{32}P incorporation was monitored by precipitation of aliquots with 10% Tricarboxylic acid and counting in LKB Optisafe scintillant in a β counter. Labelled probe was stored at 4°C for no longer than 2 weeks. Probe specific activity typically was in the range 0.5 - 1 x 10^9 cpm/µg. For use, aliquots of probe sufficient for ≥2 x 10^6cpm per filter were denatured by boiling for 5min prior to adding to the hybridisation mix.

2.5.2.2 Northern blot analysis of RNA

Formaldehyde was removed from RNA gels by rinsing in 10 x SSC (1.5M NaCl; 0.5M Na citrate pH7.0) for ≤15min. RNA was transferred onto Hybond-C nitrocellulose membrane (Amersham) by blotting for 20h with 10 x SSC, as shown in Fig 2.1. The membrane was dried between Whatman 3MM filterpaper sheets and baked for 2h at 80°C in a vacuum oven to fix the RNA.

Figure 2.1

Capillary transfer of nucleic acids from agarose gels onto membranes



Membranes were prehybridised, at 42°C for 16h with shaking, in RNA hybridisation mix (5 x SSC; 1mM EDTA; 0.5% non-fat driedmilk powder; 50% formamide; 1.25% SDS; 125µg/ml yeast RNA). Denatured ^{32}P-c-DNA probe was added to fresh hybridisation mix and the membrane hybridised at 42°C for 16h. Membranes were washed sequentially with vigorous shaking: three 15 minute low stringency washes in 2 x SSC; 0.1% SDS at RT and two 15 minute high stringency washes in 0.2 x SSC; 0.1% SDS at 56°C. After air drying membranes were exposed on Kodak XAR film.

2.5.2.3 Southern blot analysis of cDNA

Gels to be blotted were denatured for 15min in 1.5M NaCl; 0.5M NaOH then neutralised with 1M TrisHCl pH7.0; 2M NaCl for 2 x 45min with gentle shaking. DNA fragments were transferred from the rinsed gel onto nitrocellulose membranes as shown in Fig. 2.1 and baked to fix.

Membranes were prehybridised and hybridised at 65°C in DNA hybridisation mix (2.85 x SSC; 20mM phosphate pH7.0; 1mM EDTA; 0.5% milk powder; 1% SDS; 100µg/ml salmon sperm DNA). Wash conditions were were the same as for northern hybridisations except that the stringency wash was carried out at 65°C.

2.6 Cloning PCR products

2.6.1 Vectors used for cDNA cloning, sequencing and expression

pTZ18R and pTZ19R phagemid cloning vectors were purchased from Pharmacia. These vectors contain overlapping enzyme restriction site multilinkers derived from pUC18 and pUC19 plasmids which can be used for specific cloning (see Chapter 3 Fig. 3.1).

pMA5620, pUGS41S and the *E coli* / yeast shuttle vector pOGS40 were gifts from Dr. S.E. Adams, British Biotechnology Ltd, Oxford. pOGS40 plasmid contains a unique BamHI restriction endonuclease site for insertion of extraneous cDNA.
Schematic diagrams of these vectors are shown in Chapter 4 Fig. 4.1.

Vectors were digested with restriction enzymes as required and dephosphorylated by addition of 10 units calf intestinal phosphatase for the last 30min of the digestion. Cut vectors were purified by electrophoresis on 1.0% low gelling temperature (lgt) agarose gels. Bands were excised and the DNA either purified by extraction (see Sect. 2.3) or stored in lgt at -20°C.

2.6.2 Bacterial host strains (Messing, 1979; Yanisch-Perron, 1985)

pTZ18R and pTZ19R vectors were propagated in *E.coli* strain JM101 *(supE, thi, Δ(lac-proAB), [F', traD36, proAB, lacIqZ ΔM15]).*

pOGS40, pMA5620 and pUG41S vectors were propagated in *E.coli* strain JM83 *(ara, Δ(lac-proAB), rpsL(=strA), ϕ80, lacZΔM15).*

2.6.3 Media for bacterial growth

Luria-Bertani broth (LB) consisted of 1% (w/v) Bacto-tryptone; 0.5% (w/v) Bacto-yeast extract; 0.171M NaCl; pH7.5.

LB plates were made with the addition of 1.5% nutrient agar.

Selective medium was supplemented with 150μg/ml ampicillin (LB/Amp).

2.6.4 Preparation of competent *E coli* strains JM101 and JM83

A single colony selected from bacteria streaked on an agar plate was grown overnight at 37°C in 10ml LB/Amp. 1ml was innoculated into 50ml *psi* broth and grown to $OD_{550m\mu}$ = 0.3. 5ml was innoculated into 100ml *psi* broth and grown to $OD_{550m\mu}$ = 0.48 then cooled briefly on ice, centrifuged at 3000g for 10min at 4°C and the cells resuspended in 33ml ice cold Transforming buffer I (TfBI) on ice for 15min. Cells were again spun then resuspended in 4ml Transforming buffer II (TfBII) on ice for 20min. 200μl aliquots were snap frozen with liquid nitrogen and stored at -70°C.

psi Broth: tryptone 2% w/v; yeast extract 0.5%; 20mM Mg_2SO_4; 10mM NaCl; 5mM KCl.

TfBI: 35mM NaOAc; 10mM $CaCl_2$; 15% w/v glycerol; 100mM RbCl; 200mM $MnCl_2$; pH 5.9.

TfBII: 10mM morpholinopropane sulfonic acid; 10mM RbCl; 80mM CaCl2; 15% glycerol; adjusted to pH6.8 with KOH.

2.6.5 Transformation of competent *E. coli*

50-200ng vector or vector construct was added to an aliquot of bacteria, incubated on ice for 30min, heat shocked 90 seconds at 42°C then cooled briefly on ice. 900μl filtered LB was added and the tubes incubated on their sides at 37°C for 60min without shaking. Bacteria were pelleted, resuspended in 100μl LB, plated onto LB/Amp plates and incubated at 37°C overnight. Plates for growth of JM101 transfected with pTZ also contained 400mg/l X-Gal and 168mg/l IPTG (Northumbria Biologicals Ltd) for blue/white colour selection.

2.6.6 DNA ligations

2.6.6.1 End-filling and phosphorylation of PCR products

cDNA (≤1μg) was incubated with 1mM TrisHCl pH7.5, 700μM β–Mercaptoethanol, 700μM $MgCl_2$, 100mM each dNTP and 4 units Klenow DNA polymerase for 30min at room temperature to fill in any protruding ends present after PCR. The reaction was stopped by heating at 70°C for 10min then allowed to cool to 37°C. To phosphorylate the 5' termini, 1mM ATP and 20 units T4 polynucleotide kinase were added and the reaction incubated for 30min. After addition of 20mM EDTA to stop the reaction, the cDNA was phenol extracted and electrophoresed through low gelling temperature agarose. The required band was excised and the DNA either stored at -20°C in lgt agarose or extracted by absorption to silica (Sect. 2.2.2.3) prior to storage.

2.6.6.2 Ligations into pTZ18R and pTZ19R phagemid vectors

Phosphorylated PCR product was blunt-end ligated into SmaI cut dephosphorylated pTZ18R using a range of insert:vector ratios from 1:3 to 3:1 (w/w) in 'blunt-end ligation buffer' (50mM TrisHCl pH7.5; 10mM $MgCl_2$; 5% PEG_{6000}; 1mM ATP; 1mM DTT). 4 units T4 DNA ligase were added and the ligation incubated at 12°C for 16h. The reaction was stopped by heating to 70°C for 10min.

For placing inserts in the opposite orientation, selected pTZ18R clones were digested with ECoRI and HindIII restriction enzymes and the recovered purified inserts ligated into pTZ19R as above but in 'cohesive-end ligation buffer', buffer for use iwth T4 ligase, supplied by Amersham plc.

Ligation products were phenol extracted, resuspended in TCM (10mM TrisHCl pH7.0; 10mM $CaCl_2$; 10mM $MgCl_2$) and used to transform *E. coli* JM101 bacteria.

2.6.6.3 Ligation into pOGS40 expression vector

Varying ratios of dephosphorylated pOGS40 and phosphorylated PCR product, both BamHI digested, were ligated in 'cohesive-end ligation buffer' When ligating insert which had previously been excised from pTZ18R by digestion with BamHI, the insert was gel purified twice and phenol extracted prior to ligation into BamHI digested pOGS40. Ligation products were phenol extracted, resuspended in 20μl TCM and used for transformation of *E coli* JM83 bacteria.

2.6.6.4 Ligations in low gelling temperature (lgt) agarose

Lgt samples containing the required DNAs were melted at 56°C and aliquots transferred to 37°C (≤25μl total vol.). Ligation buffer and enzyme were added

and the samples incubated at 15°C for 16h. The reaction was stopped by heating to 65°C for 10min. Ligated products were hot phenol extracted, ethanol precipitated and resuspended in 20µl TCM. Aliquots were used to transform *E. coli*.

2.6.7 Selection of positive colonies

2.6.7.1 Plasmid miniprep

JM101 colonies positive for vector insert were detected on the basis of chromatic selection on LB/Amp plates containing X-gal and IPTG (Sect. 2.4.5). White colonies were grown to stationary phase in 10ml LB/Amp. Cells were pelleted and resuspended in 200µl TE pH8.0. An equal volume of phenol was added and the DNA extracted and precipitated as normal. DNA was digested with ECoRI and HindIII restriction enzymes to yield vector and insert, which were visualised on 1.2% agarose gels. Southern blot hybridisation with ^{32}P-dCTP labelled PCR product confirmed the presence of correct insert.

2.6.7.2 Colony hybridisation (Buluwela et al., 1989)

Colonies were replica plated onto three LB/Amp plates and incubated 24h. One plate was kept sterile and colony lifts were taken off the other two by carefully pressing a nylon hybridisation membrane on to the surface of the plate and marking the orientation. After 3min the filter was lifted off and placed face up on Whatman 3MM filter paper sheets soaked with 2 x SSC; 5% SDS as lysis/denaturing solution, for 2min. The filter was then microwaved for 2.5min at 650W ensuring that there was just enough liquid to prevent charring. Filters were soaked in 5 x SSC; 0.1 %SDS and hybridised following the standard method. Insert sizes in positive transformants were determined by restriction enzyme digestion of plasmid minipreps as above.

2.6.8 Large scale plasmid preparation and CsCl purification of DNA (Maniatis, 1987)

A single colony containing the required vector was grown to stationary phase in 10ml LB/Amp then subcultured in 500ml of the medium at 37°C for 16h with vigorous shaking. Pelleted cells were resuspended in 10ml Solution I (50mM glucose; 10mM EDTA; 25mM TrisHCl pH8.0; with 10mg/ml lysozyme added immediately before use) for 5min at RT. 20ml fresh Solution II (0.2N NaOH; 1% SDS) was added and left on ice for 10min. 15ml 5M potassium acetate pH4.8 was added and the tubes incubated on ice for a further 10min then centrifuged at 5000g for at 4°C 20min. Isopropanol at 6:10 (v/v) was added to precipitate the DNA. After 15min at room temperature the DNA was pelleted, washed with 70% ethanol, dried and resuspended in TE pH8.0 in a total volume of 30ml.

The DNA was purified on a CsCl gradient as follows: 30g baked CsCl and 2.4ml of 10mg/ml Ethidium Bromide were added to the DNA and the preparation centrifuged in a polypropylene tube at room temperature for 16h at 40,000rpm using a VTi50 vertical rotor. The DNA was visible as a bright orange band when viewed under UV and could easily be removed by puncturing the tube with a needle attached to a 5ml syringe and withdrawing the DNA. The EtBr was removed from the DNA by repeated extractions with iso-amyl alcohol. DNA was precipitated from the aqueous CsCl by addition of 2.5 volumes of 70% ethanol then resuspended in TE pH8.0, precipitated with ethanol and 0.25M Na acetate pH5.2, washed with 70% ethanol and finally resuspended in water.

2.7 DNA Sequencing

2.7.1 Preparation of Single-stranded DNA

A 10ml culture of transformed *E. coli* JM101 or JM83 was grown overnight in LB/Amp. 500µl was sub-cultured in 10ml 2 x YT (3.2% Tryptone; 2% yeast extract; 86mM NaCl; pH7.4 with KH_2PO_4) supplemented with 150µg/ml ampicillin until reaching $OD_{660m\mu}$ = 0.6. 400µl of this culture was infected with $2x10^8$ M13KO7 helper phage (bacteria:M13 = 1:10) and shaken vigorously at 37°C for 1 hour. 10ml 2 x YT and 73mg Kanamycin were added and the culture incubated overnight at 37°C with shaking. Cells were pelleted at 3000g for 10min. To isolate the phage, 2.5ml 20% PEG_{6000} in 3.5M NaCl was added to the supernatant, incubated on ice >30min and the precipitated particles pelleted at 11000 x g for 40min at 4°C. Care needs to be taken in removing all the PEG supernatant as traces of PEG can be inhibitory to some enzymes. The pellet was resuspended in 400µl NTE pH8.0 with vortexing, phenol extracted twice and ethanol precipitated and finally resuspended in H_2O at 1µg/µl.

2.7.2 Sequencing Reactions

All sequencing reaction were based on the dideoxy chain termination method of Sanger et al, 1977.

2.7.2.1 Sequencing using Sequenase T7 DNA Polymerase (USB)

Sequencing reactions were carried out using a Sequenase version 2.0 kit (USB Corporation) and [α-^{35}S]dATP but with slight modifications of the recommended procedure, as detailed below. Either M13 reverse primer (Pharmacia) or specific PCR primers were used. To anneal primer and template, 2-3µg single-stranded M13 cDNA, >5µg double-stranded pOGS40 or 600ng

PCR product in 7µl was boiled for 2min with 1µl primer (at a 1:1 template:primer stoichiometric ratio) and 2µl reaction buffer, then snap cooled on ice. Chain elongation and dideoxy termination reactions were carried out as per the protocol supplied with the kit except that terminations were incubated at 56°C. Reaction mixes could be stored at -20°C for short periods.

2.7.2.2 Sequencing using *Taq* Polymerase (modified from Innis et al., 1988) 6.5µl single-stranded cDNA (~3µg) was boiled for 3min with 30mM PCR primer and 2µl 10 x Ohara buffer (Sect. 2.3.7) then snap cooled. 2µl labelling mix, 7µl enzyme dilution buffer (10mM TrisHCl pH8.0 containing 0.5% Tween 20 and 0,5% Nonidet) and 1µl [α^{35}S]-dATP were added on ice and the tube warmed to 37°C. 2.5 units*Taq* polymerase were added and incubated for 2min at 37°C. Termination mixes were overlaid with oil and prewarmed at 70°C. Extension reactions were terminated by transferring 4µl of the extension reaction to each 2µl termination mix and incubating at 70°C for 2min. 2µl stop solution from the sequenase kit was added to stop the reaction.

Labelling mix: 10µM dGTP; 7.5µM dCTP; 7.5µM dTTP in 10mM TrisHCl pH8.0

Termination mixes:	ddA: 30µM each dNTP;	1.0mM ddA;	1.12mM $MgCl_2$
	ddC: 30µM each dNTP;	0.5mM ddC;	0.62mM $MgCl_2$
	ddG: 30µM each dNTP;	0.25mM ddG;	0.37mM $MgCl_2$
	ddT: 30µM each dNTP;	1.5mM ddT;	1.62mM $MgCl_2$

2.7.3 Denaturing Polyacrylamide Sequencing Gels

6% acrylamide (19:1 acrylamide:bisacrylamide w/w) gels containing 7.67M urea were run at 56°C in 0.5 x TBE using Bio-Rad Sequi-Gen apparatus. The glass plates were sealed at the base with 35ml 6% acrylamide plus 210µl 25% ammonium persulphate (AP) and 150µl TEMED (Tetramethylene-ethylene diamine). Acrylamide solutions for the slab gel were degassed under vacuum and filtered through a 0.45mM filter immediately prior to adding 100µl each of AP and TEMED and casting the gel. Gels were pre-run at 2700V for about 45min until the temperature reached 56°C. Samples were heated to 80°C for 5min. Urea was washed out of the wells using a syringe and up to 5µl of sample loaded per well. Gels were run for 2-8h depending on the resolution required. After drying under vacuum at 80°C for 2h, gels were exposed on Kodak XAR-5 film for a minimum of 12h at room temperature.

2.8 Expression of Recombinant Proteins in *Saccharomyces cerevisiae*

2.8.1 Standard Media and Solutions (Kingsman et al., 1990)

a. YEPD: 2% (w/v) peptone; 1% (w/v) yeast extract; 2% (w/v) glucose.
b. Synthetic complete-glucose medium (Sc-glc): 0.67% (w/v) yeast nitrogen base without amino acids; 1% (w/v) glucose. Appropriate amino acids added after autoclaving.
c. Sc-glc/gal medium: 0.67% (w/v) yeast nitrogen base without amino acids; 0.3% (w/v) glucose; 1% (w/v) galactose. Appropriate amino acids were added after autoclaving.
d. Glycerol stocks: yeast strains were stored at -70°C in Scglc;20% glycerol.
e. Amino acids: Tryptophan, uracil and leucine all used at 20mg/l.
f. Agar plates: 2% (w/v) nutrient agar was added to the medium prior to autoclaving.
g. TEN buffer: 10mM TrisHCl pH7.4; 2mM EDTA; 140mM NaCl.
h. Acid washed glass beads: Glass beads, 40 mesh (BDH), washed in concentrated sulphuric acid, rinsed 10 times in tap water and ten times in distilled water, dried and baked at 150°C for 2h.

2.8.2 Yeast Strains

The protease deficient *Saccharomyces cerevisiae* strain BJ2168 (*a, leu2*$^{=}$*, trp1*$^{-}$*, ura3-52, prb1-1122, pep4-3, pcr1-407, gal2*) (Jones, 1991) was transformed to leucine and uracil independence with pOGS40 plasmid constructs in the presence or absence of pUGS41S plasmid using the method of Hinnen et al., 1978.

2.8.3 Yeast Transformation

All manipulations were carried out aseptically.

Untransformed yeast from glycerol stocks stored at -70°C was inoculated into 100ml YEPD, grown overnight at 30°C then subcultured to a density of $\geq 1.5 \times 10^7$ cells/ml (OD_{600nm} ~0.7). Cells were harvested at 3.500g for 5min at room temperature, washed with 20ml 1M sorbitol and resuspended in 10ml 1M sorbitol pH5.6. 200µl glusulase (du Pont) was added and incubated at 30°C for 2h with occasional gentle shaking. The extent of spheroplasting was determined by mixing 100µl cell suspension with 1ml water and observing under a light microscope. Spheroplasts remain intact in sorbitol but burst in water. The very fragile spheroplasts were carefully washed twice with 10ml sorbitol and once with 10ml STC solution (1M sorbitol; 10mM $CaCl_2$; 10mM TrisHCl pH8.0)

then resuspended in 1ml STC. 2-3μg DNA, (or 2-3μg of each DNA construct in the case of double transformants with pUG41S vector), was added to 100μl competent cells, mixed by gentle flicking and incubated at room temperature for 15min. The total volume of DNA added must be less than 15μl in order to maintain osmolarity. 1ml 44% PEG_{4000} was added, mixed by inversion and gently flicking and left at room temperature for 10min. Cells were harvested gently using the pulse button on a microfuge and resuspended in 1ml 1M sorbitol then added to 20ml warm (not >48°C) regeneration agar (Sc-glc agar with added 1M sorbitol and amino acids). After gentle mixing, the agar was poured into petri dishes and incubated inverted at 30°C until colonies became visible (5-10days). Individual transformants were streaked on selective Sc-glc plates containing appropriate amino acids and re-incubated.

2.8.4 Storage of Yeast Transformants

50ml Sc-glc medium plus amino acids, was inoculated with a single colony and incubated with vigorous shaking at 30°C until a concentration of 2-4 x 10^7 cells/ml was achieved. An equal volume of 40% glycerol was added and 1ml aliquots frozen at -20°C for 2h before being transferred to -70°C for long term storage.

2.8.5 Validation of Glycerol Stocks for P1 or P1-Fusion Protein

2.8.5.1 Constitutive Expression of P1 vlps (pMA5620 derived plasmid)

50ml Sc-glc plus 20mg/ml each tryptophan and uracil was inoculated with a glycerol aliquot and shaken vigorously at 30°C for 2-3 days. 5 x 10^8 cells were harvested at 3500 x g for 5min and resuspended in 1ml TEN buffer. 1g acid washed glass beads were added and the cells disrupted by vortexing 3 x 1 minute, cooling on ice for 1 minute in between. 10μl aliquots were analysed by electrophoresis through 10% PAGE gels and blotting against anti-P1 antiserum (Sect. 2.9).

2.8.5.2 Galactose Induced Expression of IL-1:P1 vlps (pOGS40 constructs ± pUG41S plasmid)

50ml Sc-glc plus 20mg/ml tryptophan was innoculated with a glycerol aliquot and incubated at 30°C for 2-3 days. Cells were spun down, resuspended in 50ml Sc-glc/gal with tryptophan and shaken a further 24h. 5 x 10^8 cells were harvested and treated as above.

2.8.6 Large Scale Culture of Recombinant Proteins

2.8.6.1 Constitutive Expression

100ml Sc-glc plus tryptophan and uracil was inoculated with the selected transformant and shaken vigorously on a flat bed rotator at 30°C for 48h. 2 x 1l medium were inoculated with 50ml preculture and grown a further 24h to (~5 x 10^7cells/ml). Cells were harvested, washed four times with SDW and once with TEN then either used immediately or stored as a pellet at -20°C in Falcon tubes.

2.8.6.2 Inducible Expression

100ml Sc-glc plus 20mg/ml tryptophan was innoculated with the selected transformant and shaken vigorously at 30°C for 48h. The preculture was inoculated into 1l medium and grown until cell numbers were about 4 x 10^7 per ml. 250ml of the culture was innoculated into each of 4 x 1l Sc-glc/gal plus tryptophan and shaken for 48–66h depending on the transformant. Cells were harvested, washed four times with SDW and once with TEN, then either used immediately or stored as a pellet at -20°C.

2.8.7 Purification of Recombinant Proteins

2.8.7.1 Preparation of vlps

Harvested cells were transferred to 30ml Corex tubes (5-7ml cells per tube) and resuspended in 4ml ice cold TEN buffer. 5ml acid washed glass beads were added and the tubes vortexed for 10 x 30sec periods interspersed with 30second periods of cooling on ice. The suspension was centrifuged at 2000 x g for 5min and the supernatant transferred to 30ml Oakridge tubes on ice. The pellet was revortexed with 4ml fresh TEN buffer, respun and the supernatant added to the Oakridge tubes. The cracking procedure was repeated with a further 3ml TEN buffer. Pooled supernatants were centrifuged at 30,000 x g for 20min. The resultant supernatant was layered over 60% (w/v) sucrose/TEN and centrifuged at 100,000 x g for 90min. The interface band and cushion were either collected separately or pooled, depending on the protein, and dialysed overnight into fresh TEN buffer. Any flocculant precipitate was removed from the dialysate by centrifugation at 13,000 x g for 20min.

2.8.7.2 Purification of vlps by Sucrose Density Gradient Centrifugation

Sucrose gradients in TEN buffer were prepared by carefully layering sequential 8ml volumes of cold 45%, 35%, 25% and 15% sucrose into 40ml Beckman ultraclear centrifuge tubes and allowing a linear gradient to develop by diffusion at 4°C overnight. Immediately before use, a 2ml 60% sucrose cushion was layered under the gradient using a pasteur pipette. Gradients were loaded with up to 4ml of vlp containing dialysate and centrifuged in an SW28 rotor for 3h at 25,000rpm..

Sequential 2ml fractions were removed from the top of the gradients and stored at 4°C. The distrubution of vlps across the gradient was assessed by running 5-10µl samples from each fraction on 10% SDS/PAGE gels and staining with coomassie blue (see Sect. 2.9.2). Protein concentrations in each fraction were determined by Bio-Rad assay (see Sect. 2.9.4).

2.8.7.3 Cleavage of Fusion Proteins with Restriction Protease Factor Xa (FXa)

Selected gradient fractions were dialysed into Factor Xa buffer (10mM $CaCl_2$; 100mM TrisHCl pH7.6) at 4°C. Expressed protein was released from vlps by digestion at 25°C with FXa (1:100 w/w) in the presence of added 0.02% DOC (NaDeoxycholate) and 0.02% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate). Cleaved vlps were removed by centrifugation at 40,000 x g for 90min and the recombinant protein dialysed into PBS containing 0.1% Na azide and stored at 4°C or -70°C.

2.8.7.4 Purification of Recombinant Proteins by Ion Exchange HPLC

Cleaved vlp preparations were dialysed into 20mM TrisHCl pH8.0. ≤5ml samples at ~4µg/ml were purified by HPLC using an ion exchange column at a flow rate of 1ml/min. Separation was achieved by use of a 0-300mM NaCl gradient in 20mM TrisHCl pH8.0. 500µl fractions were collected. 50µl aliquots of these fractions were precipitated overnight with 300µl cold acetone and analysed by 15% SDS/PAGE gels (Sect. 2.9). Selected fractions were iodinated or biotinylated (Sect. 2.14.1.2).

2.9 Analysis of Recombinant Proteins by SDS-PAGE

2.9.1 Sample Preparation

Samples and molecular weight markers (Pharmacia, used at 6µg per well), were prepared by boiling for 3min in sample buffer (150mM β-mercaptoethanol; 15% glycerol; 0.05% SDS; 0.005% (w/v) bromophenol blue; 50mM TrisHCl pH6.8).

2.9.2 Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were analysed by discontinuous polyacrylamide gel electrophoresis run under reducing conditions (Laemmli, 1970) using Bio-Rad Mini-protean II vertical slab gel apparatus.

A stock solution of 30% acrylamide:0.8% bisacrylamide was diluted as required in 250mM TrisHCl pH8.7 containg 1.3mM EDTA and 0.1% SDS. Gels were polymerised by addition of 0.5% ammonium persulphate and 0.05% Temed. The stack gel solution consisting of stock acrylamide diluted to 3.5% with 0.145M TrisHCl pH6.8 containing 0.1% SDS, 0.05% ammonium persulphate

and 0.1% TEMED was overlaid on the separating gel. After gel polymerisation, samples were electrophoresed at 200V in 25mM TrisHCl containing 6mM EDTA, 0.1% SDS and 156mM glycine.

2.9.3 Detection of Protiens within PAGE Gels

2.9.3.1 Staining PAGE Gels with Coomassie Blue

Gels were stained with coomassie brilliant blue G-250 or coomassie blue R (0.25% (w/v) coomassie blue; 20% methanol; 5% acetic acid), destained with 20% methanol; 5% glacial acetic acid and dried onto Whatmans 3MM filterpaper under vacuum at 80°C.

2.9.3.2 Staining PAGE Gels with Silver Nitrate

Gels were rinsed with de-ionised water then fixed sequentially with; (i) 50% methanol; 10% acetic acid for 15min, (ii) 5% methanol; 7% acetic acid for 30min, (iii) 10% glutaraldehyde for 30min. Fixed gels were washed in several changes of distilled water over a few hours. Gels were stained with 0.1% w/v silver nitrate for 15min, rinsed briefly with water and developed with 2.6% w/v sodium carbonate in 0.02% formaldehyde. The reaction was stopped by addition of solid citric acid. Gels were washed well with water, fixed in 10% Ilfofix for exactly 1 minute, rinsed briefly and dried under vacuum at 80°C.

2.9.4 Estimation of Protein Concentration

Vlp concentrations were determined using a dye-binding assay (Bradford, 1976) obtained from Bio-Rad Laboratories.

Where recombinant protein concentrations were below the limits of detection of the assay, sample dilutions were run alongside protein standards on SDS-PAGE gels and the protein concentrations estimated from the relative staining intensities on coomassie and silver stained gels.

2.9.5. Electroblotting PAGE Gels

After electrophoretic separation on SDS-PAGE gels, proteins were transferred to Amersham Hybond C nitrocellulose membranes using a semi-dry electroblotter (Ancos, Denmark) according to Khyse-Andersen (1984). Transfer was achieved by electroblotting for 45min at 120mA between sheets of Whatman 3MM paper soaked in 25mM TrisAc; 20% methanol. The molecular weight marker track was cut off, stained with 5% amido black and destained with 50% methanol; 5% acetic acid.

2.9.6 Western Blot Analysis of Proteins

Membranes were 'blocked' with 5% non-fat dried milk in PBS for 4h at room temperature then incubated with primary rabbit antibody diluted in 1% milk/PBS for a further 20h at 4°C. Blots were washed with several changes of 1% milk/PBS over 30min at room temperature. The primary antibody was detected by incubation with anti-rabbit IgG alkaline phosphatase conjugate (Sigma) in 1% milk at room temperature for 60min. Excess reagents were removed by washing with several changes of 1% milk/PBS then twice with 0.1M TrisHCl pH9.5. Immunoblots were developed in 0.1M TrisHCl pH9.5 containing 0.2mg/ml Nitroblue tetrazolium (NBT), 0.1mg/ml Bromo-chloro-imidolyl-phosphate toluidine salt (BCIP:- stock solution 10mg/ml in dimethyl formamide, stored at -20°C) and 0.2mM $MgCl_2$. The reaction was stopped with water and blots were air dried.

2.10 Transmission Electron Microscopy (TEM)

2.10.1 Preparation of Yeast Cells for TEM

Fixation of yeast cells: 25ml of stationary phase yeast culture was centrifuged at 2500 x g for 5min at 4°C. The cell pellet was resuspended in 5ml cacodylate/glutaraldehyde buffer (0.1M Na cacodylate pH6.8; 5mM $CaCl_2$; 3% glutaraldehyde), incubated 30min at room temperature, spun down, resuspended in 10ml buffer and mixed on a rotator at 4°C for 20h. Cells were washed twice with 10ml pretreatment buffer (0.2M TrisHCl pH8.1; 20mM EDTA; 0.1M βmercaptoethanol, spun down and resuspended in 5ml 0.2M phosphate-citrate buffer pH5.8 containing 250µl glusulase (du Pont). After 60min at 30°C cells were checked for spheroplasting by resuspension in water. Spheroplasted yeast cells were washed twice with 10ml phoshate-citrate buffer and resuspended in 5ml of this buffer. Post fixation, the cells were incubated in cacodylate buffer containing 2% osmium tetroxide on ice for 60min, rinsed with water, incubated in 2% aqueous uranyl acetate at 20°C for 60min. Samples were dehydrated through graded acetone solutions and embedded in araldite resin. Ultrathin sections were examined using a Phillips TEM 400 operating at 100kV.

2.10.2 Negative Staining of Ty-vlps for TEM

A drop of vlp containing sample was placed on a plastic coated TEM grid and allowed to dry partially for 15 seconds. It is important that the vlps be allowed to adhere to the grid without the sample drying out. The grid was washed 3 times with 1 drop of water and partially dried. The sample was fixed with 1 drop of 2% aq. uranyl acetate. Samples were examined directly by TEM.

2.11 Assessment of Recombinant IL-1 Bioactivity

2.11.1 *In vitro* Thymocyte Co-mitogen Assay

Thymocytes were gently teased out of a fresh thymus into RPMI medium supplemented with 10% foetal calf serum, 5mM L-glutamine, 100 U/ml benzylpenicillin and 100 U/ml streptomycin. The suspension was depleted of cell clumps by sedimentation under gravity for 5min. Cells were centrifuged at 1500 x g for 5min at 4°C, washed twice with RPMI;10%FCS medium plus 50mM β-mercaptoethanol ($RPMI_{10.ME}$) and resuspended in $RPMI_{10.ME}$ at $4x10^6$ cells/ml. IL-1 containing samples were diluted in PBS. Phythemagglutinin (PHA) as co-mitogen was diluted in $RPMI_{10.ME}$. 200µl cultures containing 1 x 10^6 cells, 100µl sample and 9µg/ml PHA were established in triplicate in 96 well tissue culture plates (Nunculon, Denmark). Negative controls were established by substituting sample with PBS or PHA with medium. Proliferation assays were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. After 48h, assays were pulsed with 1µCi ^{3}H-thymidine (sp. activity 2Ci/mMole, Amersham) for 20h. Cells were collected onto glass-fibre filter paper using a semi-automated harvester and incorporated ^{3}H-thymidine estimated by liquid scintillation counting.

For neutralisation assays, rIL-1 was pre-incubated in the culture plates at 37°C for 1h (as recommended for comercial anti-IL-1 antisera), either with or without heat inactivated polyclonal antiserum in a total volume of 100µl. Thymocytes and PHA were then added and the assay carried out as above. Negative controls were established by pre-incubating antiserum with medium.

2.11.2 *In vitro* Cartilage Degradation Assay

1mm x 2mm discs, cut from fresh ovine xiphoid cartilage were washed with DME medium supplemented with 5% foetal calf serum and 100 U/ml penicillin and streptomycin (DME_5). Discs were cultured individually in DME_5 in 96 well culture plates (Nunculon) at 37°C in a humidified atmosphere of 5% CO_2/95% air. After 40h, the medium was replaced with 100µl fresh medium and quadruplicate cultures established by addition of 50µl sample in PBS. Negative controls were established by substitution of sample with PBS. Release of glycosylaminoglycans (gags) was measured after 30h incubation. An aliquot of culture supernatant was added to 0.0018% (v/v) dimethylmethylene blue dye in formate buffer (0.21% (v/v) formic acid; 0.2% (w/v) Na formate; 0.5% ethanol) and $OD_{535m\mu}$ read within 10min. The amount of gags present was read off a standard curve constructed using fresh shark chondroitin sulphate solutions.

2.12 Production of Polyclonal Rabbit anti-ovine-IL-1 Antiserum

Polyclonal antisera were raised in rabbits against recombinant ovine IL-1, yeast P1 and Factor Xa. Rabbits were injected with 10-20μg antigen in complete Freund's adjuvant and boosted at intervals with 5-10μg antigen in incomplete Freund's adjuvant. Animals were exsanguinated by cardiac puncture. Sera were heat inactivated by incubation at 56°C for 10min and stored in aliquots at -20°C.

2.13 Ovine Afferent Lymph

2.13.1 Antigenic Stimulation of Sheep and Cannulation of the Afferent Lymph Ducts

Pseudo-afferent lymphatic cannulations were performed by Dr. John Hopkins (Department of Veterinary Pathology, University of Edinburgh) as described by Hall, 1967. Prefemoral lymph nodes were excised at least eight weeks prior to cannulation to allow the afferent and efferent lymphatic vessels to re-anastamose after removal of the node. Sheep were simultaneously immunised with 1mg ovalbumin injected over two sites intramuscularly in a 1:1 mixture of PBS and complete Freund's adjuvant or 9% Alum and subsequently boosted with 500μg ovalbumin, administrated intravenously in PBS after 4-6 weeks. Cannulated sheep were kept in standard pattern metabolism cages and fed water and hay *ad libitum.*

2.13.2 Establishing a Positive Response to Ovalbumin by *in vitro* Proliferation of PBMC from Ovalbumin Primed Sheep

Peripheral blood from ovalbumin-primed sheep was obtained by venipuncture and collected into preservative free heparin at a concentration of 10 units per ml of blood. White blood cells, buffy coated by centrifugation at 1300 x g for 20min at 20°C were diluted 1:3 with sterile PBS and centrifuged over lymphoprep (Nyegaard, Norway) at 850g for 20min at 20°C. Mononuclear cells isolated from the interface were washed once with fresh PBS and twice in $RPMI_{10.ME}$. Cells were harvested by centrifugation at 250 x g for 4min at 4°C between each wash step. 1 x 10^5 cells/well in $RPMI_{10.ME}$ were cultured in 96-well flat bottomed micro-culture plates (Nunclon, Denmark) with addition of various concentrations of antigen in a total volume of 200μl. Cultures were established in triplicate. Positive and negative controls were established by substituting antigen with either Con A (20μg/ml; 6.7μg/ml; 2.2μg/ml) or medium respectively. The proliferation assays were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air for 5 days and pulsed with 1μCi ^{3}H-thymidine (specific activity 2Ci/mMole, Amersham) over the final 7h. Cells

were collected onto glassfibre filter paper using a semi-automated harvester and ^{3}H-thymidine estimated by liquid scintillation counting.

2.13.3 Isolation of Afferent Lymph Cell Populations

Pseudoafferent lymph was collected into sterile 250ml plastic bottles containing $2.5x10^{3}$ units of heparin and $2.5x10^{4}$ units each of penicillin and streptomycin. Cells were washed and resuspended in cold Iscoves serum free medium. 2ml medium containing $\leq 2 \times 10^{8}$ cells was layered over a discontinouus gradient of 2ml 20% metrizamide (Nycomed, Oslo, Norway) and 3ml 14.5% metrizamide in Iscoves and centrifuged at 1500 x g for 20min at 4°C. The upper and lower interface populations were collected separately, washed twice with 20ml cold PBA (PBS; 0.1% BSA; 0.1% Na azide) and resuspended in PBA at 8×10^{6} per ml.

2.13.4 Analysis of Cell Populations by Cytochemical Staining

2.13.4.1 Preparation of Cytospins

Cells were applied to acid washed poly-L-lysine coated glass slides by centrifugation at 350g for 6min at room temperature. Cells were fixed and the slides either used immediately or stored in an airtight container at -20°C.

2.13.4.2 Giemsa Stain

Cytospun cells were fixed in cold acetone then stained for 5min in 2% (v/v) Giemsa stain. Slides were destained in water.

2.13.4.3 Leishman's Stain

(Darmady and Davenport, 1963)

Cytospun cells were acetone fixed. A stock solution of Leishmans stain (1% Grubler's methylene blue in H_2O; 0.5% Na carbonate; 0.1% Grubler's eosin extra B in methyl alcohol) was diluted with 2 vols H_2O for use. Slides were stained for 5min and destained with water.

2.13.4.4 Acetylcholinesterase Stain for Ovine Langerhans' Cells

(Adapted from Hollis and Lyne, 1972).

Cytospins were acetone fixed. Slides which had been stored at -20°C were incubated in 0.22M sucrose at room temperaturefor a few minutes prior to staining. Cytospins were incubated at 37°C for 70min in incubation medium (see below), washed twice with distilled water then incubated for 5min at room temperature in 10mM ammonium sulphide. Slides were washed twice with distilled water, counterstained with methylene blue for 75sec, rinsed and mounted.

Incubation medium: (It is important that the ingredients are added in order)

50mg acetylthiocholine iodide
65ml sodium hydrogen maleate buffer pH7.2 (32mM NaOH; 5.3mg/ml maleic acid)
5ml sodium citrate (24.9mg/ml)
10ml $CuSO_4$ (7.47mg/ml)
10ml water
10ml potassium ferricyanide (1.65mg/ml)
7.5g sucrose

Methylene blue: 3ml saturated methyl blue in alcohol plus 10ml 0.01% KOH in water.

2.13.4.4 Non-specific Esterase Stain for Macrophages

(Hudson and Hay, 1989)

Cytospun cells were fixed at 4°C for 2min in 30mM Phosphate pH6.6 containing 9.25% formaldehyde and 45% acetone then washed with water. Fixed cells were incubated at 37°C for 45min with activated hexazotised pararosaniline (1.5% w/v pararosaniline; 75mM HCl; 0.12% w/v Na nitrite in 67mM phosphate pH5.8) containing 0.025% α-naphthyl acetate (2.5% w/v in acetone) then counterstained for 10min with 0.4% aqueous methylene green. Excess stain was removed by washing with water.

2.13.5 Analysis of Cell Populations by Immunofluorescence

Surface markers for macrophages and afferent lymph dendritic cells were detected by incubation with mouse anti-sheep monoclonal antibodies which were available in the department. These were used in order to be able to distinguish between these two cells types within afferent lymph populations.

2.13.5.1 Immunofluorescence Analysis

Fluorescence analysis was carried out using a Becton Dickinson FACScan which allowed measurements to be made on homogeneous populations of cells by setting electronic gates in order to 'isolate' cells with particular forward and side scatter profiles (FSC and SSC respectively). 10^4 cells were analysed per sample. The fluorescence profile is displayed as a histogram representing fluorescence intensity vs. relative cell number. Positive flourescence due to binding of a monoclonal antibody to its specific ligands is indicated by a shift to the right relative to background fluorescence intensity.

2.13.5.2 Monoclonal Antibodies

Monoclonal antibodies used in this study are detailed in Table 2.1. Isotype and specificity are included.

TABLE 2.1

Monoclonal antibodies used for assessing the surface phenotype of alveolar macrophages and afferent lymph cell populations

Antibody	Specificity	Isotype	Reference
SBU T4	ovine CD4	IgG_{2a}	Maddox et al., 1985
SBU T8	ovine CD8	IgG_{2a}	Maddox et al., 1985
CC14	ovine CD1	IgG_1	MacHugh et al., 1988
VPM5	ovine CD1	IgM	Bujdoso et al., 198
VPM13	ovine μ-chain	IgM	unpublished
VPM32	ovine	IgG_{2a}	Gonzales, 1991
VPM54	ovine DRα	IgG_1	Dutia et al., 1993
VPM65	ovine CD14(?)	IgG_1	V Gupta, personal communication
1D10	EV1 p25	IgG_1	Reyburn et al., 1992

2.13.5.3 Immunofluorescence Staining

Cell surface phenotype was assessed using 2 x 10^5 cells. Washed cells were resuspended in 25μl PBA and incubated with 25μl primary antibody at the appropriate dilution for 30min on ice. Saturated supernatants were used neat and ascites fluid at a dilution of 1 in 500. Unbound antibody was removed by washing with 3 x 1ml PBA. Biotinylated antibodies were detected by incubating with phycoerythrin conjugated to streptavidin (Serotec, Oxford). Non-biotinylated antibodies were detected by incubation with 25μl of a 1 in 100 dilution of FITC conjugated $F(ab)_2$ fragment of rabbit anti-mouse immunoglobulin (Dakopatts, Denmark) for 30min on ice. Cells were washed, resuspended in 600μl PBA and analysed by flow cytometry.

2.14 IL-1 Receptor Studies

2.14.1 Labelling Recombinant Proteins

2.14.1.1 Iodination of rIL-1

IL-1α and IL-1β were iodinated following the Iodobead procedure of Markwell, 1982. 1μg protein in either 1ml PBS or 1ml 20mM TrisHCl adjusted to pH7.0, was incubated for 15min at room temperature with 1 activated Iodobead in a 1.8ml screwtop Eppendorf tube. Unincorporated isotope was removed by extensive dialysis against PBS:0.1% azide at 4°C. ^{125}I incorporation was estimated from 10% TCA precipitable counts pre and post dialysis. 1μl samples were added to 500μl PBA on ice. 500μl ice cold 20% TCA was added and the samples vortexed then left to precipitate on ice for 30min. Samples were filtered through Whatman GF/C discs and washed with 50ml cold 10% TCA under suction followed by 20ml absolute ethanol. The precipitated radioactivity on dried filters was counted in a γ-counter. Purity of the samples was visualised by autoradiography of dried SDS/PAGE gels. Labelled protein was stored at 4°C. Control p1 preparations were similarly iodinated.

2.14.1.2 Biotinylation of rIL-1

IL-1β samples at ~1μg/ml were dialysed into 0.1M $NaHCO_3$ pH8.0 at 4°C. To this was added 100ng biotin in 50μl DMSO and the solution rotated for 4h at room temperature. The biotinylated protein was dialysed extensively against PBS then 0.1% Na azide added and the preparation stored in the dark at 4°C. Because of the very small amounts of protein available for biotinylation, losses could only be estimated from figures obtained during iodination parallel iodination procedures.

The purity and extent of biotinylation of IL-1β was assessed by enhanced chemiluminescence using an Amersham kit. SDS-PAGE gels were electroblotted and blocked then incubated with biotin:phycoerythrin:streptavidin complex. The membrane was well washed with PBS containing 0.1% bovine serum albumin then placed in a suitable container in a photographic darkroom. To develop the blot, 1ml each of solutions A and B were mixed and placed on the membrane for exactly 1min then rinsed off with water. The blot was covered with clingfilm and exposed on Amersham ECL Hyperfilm for 1-2min. The Hyperfilm was developed as normal.

2.14.2 Detection and Quantitation of IL-1 Receptors on Dendritic Cells and Lymphocytes within Afferent Lymph and on Alveolar Macrophages

2.14.2.1 Use of ^{125}I-rIL-1 Binding to Estimate of IL-1 Receptor Density

2.14.2.1.1 Analysis of Total ^{125}I-IL-1 Bound to Isolated Cell Populations

Isolated cell populations were resuspended in PBA and incubated with ^{125}I-IL-1, either with or without an excess of unlabelled IL-1, in a total volume of 100µl in 1.8ml screw-cap Eppendorf tubes. Samples were incubated on a rotating mixer for 2.5h at 4°C then washed with three 1.8ml volumes of ice-cold PBA, resuspended in 100µl PBA and layered over 300µl ice-cold phthalate oil (dibutyl phthalate:bis(-ethylhexyl) phthalate 1.5:1 v/v) in 0.3ml microsedimentation tubes (Sarstedt Ltd., Leicester). Sample tubes were microfuged at 13500rpm for 110sec at 4°C then frozen in a dry ice/ethanol bath and the tip containing the cell pellet cut off and counted in a γ counter. IL-1 receptor characteristics were determined by Scatchard analysis of bound ^{125}I-IL-1 (Scatchard, 1949).

2.14.2.1.2 Analysis of Bound ^{125}I-IL-1 at a Single Cell Level by Radiographic Exposure of Cytospins

Samples were prepared and incubated as above then washed with five 1.8ml volumes of ice-cold PBA and cytospun at 300g for 6min onto acid-washed glass slides. Samples were fixed with cold acetone and the slides dipped in Amersham LM-1 emulsion diluted 1:2 with water. Exposure times varied depending on the expected signal. Samples were developed by successive dipping as follows: 3min in Kodak D19 developer (reconstituted as per the manufactures instructions and diluted 1:1 (v/v) with water prior to use); 5 seconds in 1% glacial acetic acid; rinse with water; 6min in Ilfofix (diluted 1:1 with water prior to use). Slides were gently rinsed in water over 30min, dried and mounted.

Non-specific esterase staining of cytospins was done prior to dipping the slides in emulsion whereas Giemsa staining was done after developing the exposed slides.

2.14.2.2 Detection of IL-1 Receptors by FACScan Analysis of Bound Biotinylated IL-1β

All incubations were carried out on ice. 2 x 10^5 cells in PBA were incubated for 60min with up to 20ng biotinylated-IL-1 in a total volume of 50µl. Unbound material was removed by washing three times with 1ml PBA. Cells were

incubated with 25µl of a 1 in 400 dilution of streptavidin/phycoerythrin conjugate (Serotec, Oxford) for 20min, washed three times and resuspended in 600µl PBA. Bound IL-1 was detected and analysed using a Becton Dickinson FACScan with gates set as determined in Sect. 2.11.5.

2.15 Equations Utilised During this Thesis

1. Relative centrifugal force (g) = $1.12r(RPM/1000)^2$ where r is radius of the rotor.

2. Hexanucleotide frequency in random sequence DNA is 1 in 4^6 (4096) base pairs.

3. For estimating the melting temperature of oligonucleotide / cDNA annealing:

   $T_m = 3GC + 2AT$
   with a reduction of 1°C for each expected nucleotide mismatch.

4. For estimating growth of *E. coli* : 8×10^8 cells/ml = 1.0 OD_{660nm}

5. For estimating growth of *S. cerevisiae* : 5×10^7 cells/ml = 1.5 OD_{600nm}

6. Scatchard analysis of membrane receptor expression (Scatchard, 1949);

   Data from ^{125}I-Ligand binding to cells was plotted as 'Bound/Free cpm' vs. 'pg Ligand Bound'.
   Receptor sites/cell was calculated from the x intercept and the affinity constant from the slope of the curve.

CHAPTER 3

CLONING AND SEQUENCING OVINE INTERLEUKIN-1α AND INTERLEUKIN-1β cDNA

INTRODUCTION

At the time this study was initiated, human IL-1α and IL-1β and rabbit IL-1α genes had been cloned and fully sequenced (Furutani et al., 1986; Clark et al., 1986; Bensi et al., 1987) showing that both genes consisted of seven exons with six introns, spanning primary transcripts of roughly 10,206bp (IL-1α) and 7,008bp (IL-1β). Restriction mapping of human and bovine genomic DNA had shown both genes to exist as single copies (Furutani et al., 1986; Maliszewski et al., 1988). A number of IL-1 cDNA sequences had been determined and there appeared to be a large measure of cross-species homology. Despite this homology, preliminary work had shown that reagents developed from human rIL-1 were fairly ineffectual for detecting ovine IL-1. This had also been shown to be the case for bovine IL-1. The bovine IL-1 cDNA sequences, which had only just been published (Maliszewski et al., 1988), might have been expected to be much closer to the ovine but the reagents were not available. As my intention was not only to generate probes for the study of ovine IL-1α and β production at a molecular level, but also to express the recombinant proteins, the most obvious direction in which to proceed was to clone the ovine IL-1 cDNA sequences which would subsequently be used for expression purposes.

Methods available for isolating and cloning specific nucleotide sequences included screening of genomic or cDNA libraries with appropriate probes and the comparatively new technique of polymerase chain reaction (PCR), which utilised sequence specific primers and the heat stable *Taq* polymerase enzyme to amplify DNA sequences (Mullis and Faloona, 1987). Library screening methods were in general use and had been used for identifying all previous IL-1 sequences. In view of the immediate requirement for the IL-1 coding sequences for expression purposes, genomic screening was not considered to be applicable in the first instance. Screening genomic DNA for IL-1 sequences which would yield information on the genomic organisation of the IL-1 genes, including

intron/exon boundaries and specific IL-1 promoter and regulatory sequences, could be done later but was not intended to be part of this thesis. The IL-1 mRNA sequences could be obtained by either by screening ovine cDNA libraries with the human or murine cDNAs as probes or alternatively by PCR.

PCR seemed to provide the most direct method by which IL-1 sequences could be efficiently and uniquely amplified. The fidelity of *Taq* polymerase had to be taken into consideration. It had been estimated that around 10 errors per 15000 bases could be introduced during the polymerase step of the reaction (Saiki et al., 1988). By obtaining a consensus sequence from a number of PCR-derived clones this problem should, however, be obviated. The availability of suitable sequences from which PCR primers could be chosen was also an important consideration. A number of points had to be taken into account such as primer length, total GC content, the requirement for the 3' end of the primer to be GC rich if possible, potential for annealing between the two primers and potential for cross-hybridisation to cDNAs not encoding IL-1. Published IL-1 sequences showed regions of homology which were conserved across species and from which suitable PCR primers could be chosen. It was therefore decided to employ this technique for amplification of the ovine IL-1 cDNAs.

Alveolar macrophages were chosen as the source of RNA, from which cDNA could synthesised, because they produce large amounts of IL-1 mRNA on stimulation with LPS and an abundant and relatively pure cell population is easily obtainable by bronchoalveolar lavage at postmortem. PCR products amplified from first strand cDNA populations could be readily purified for ligation into cloning vectors.

Of the numerous vectors available, the multifunctional phagemids pTZ18R and pTZ19R (illustrated in Fig. 3.1), which can be replicated in *E. coli* JM101 host cells (Mead, 1986), exhibit a number of features useful for cloning and sequencing PCR products;

(i) The two vectors are identical apart from the presence of multiple cloning sites derived from the *lac Z'* gene of the pUC plasmid (Yanisch-Perron et al., 1985). This polylinker consists of a number of overlapping unique restriction enzyme sites, which enables cDNA fragments with a range of termini to be inserted into appropriately digested vector. pTZ18R and pTZ19R contain this multiple cloning site in reverse orientations which allows an inserted fragment to be specifically excised from the one vector and oppositely orientated in the other;

(ii) IL-1 mRNA was expected to be <2kb, a size easily accepted by the vector;

(iii) the small size of the vector, 2.9kb, should allow high transformation efficiency;

(iv) the presence of an ampicillin resistance gene confers the ability to select for specifically transfected bacteria on solid media containing ampicillin;

(v) the *lac Z'* gene flanking the multiple cloning site of the vector allows easy identification of colonies positive for insert cDNA. The presence of an intact *lac Z'* gene leads to blue colonies on plates containing the appropriate chromogenic substrate, whereas disruption of the gene by inserted cDNA leads to white colonies;

(vi) superinfection of JM101 transformants with the helper bacteriophage M13KO7 allows single stranded cDNA to be synthesised from the f1 origin of replication within the vector. Because of the reverse orientations of the multiple cloning sites with respect to this origin of replication, each strand of the insert DNA can be separately synthesised;

(vii) 'Universal' and 'Reverse' sequencing primers are available (Pharmacia) which anneal to upstream oligonucleotide sequences on either side of the multiple cloning site. These primers can be used to obtain sequence from each strand of double stranded cDNA inserts. The 'Reverse' sequencing primer can also be used to sequence single stranded cDNA preparations.

For expression purposes, the yeast Ty system was chosen. This system utilises the pOGS40 expression vector (a gift from Dr. S. Adams, British Biotechnology Ltd.) (Fig. 3.2.a) which is an *E. coli* / yeast shuttle vector containing modules for selection by ampicillin resistance in *E. coli* and for leucine selection in yeasts auxotrophic for leucine biosynthesis. Full details of this expression system are given in Chapter 4. The pOGS40 vector contains a unique BamHI restriction site for insert ligation within the 3' end of a sequence coding for the yeast p1 protein. A number of other restriction sites are present within the vector which can be utilised for determining the orientation of the inserted IL-1 cDNA. In this system, recombinant IL-1 would be produced as a p1:IL-1 fusion protein from which the rIL-1 would subsequently be cleaved by the action of activated coagulation factor Xa (FXa). Sequences encoding the FXa recognition site have therefore to be inserted between the p1 and IL-1 sequences. Sequences encoding BamHI restriction endonuclease sites are also required at either end of the cDNA to be inserted into the vector. These sequences can be most efficiently appended to the IL-1 coding sequences during PCR, by using tailored IL-1 primers which include the required additional nucleotides and/or a BamHI site at their 5' ends.

FIGURE 3.1

pTZ18R and pTZ19R Vectors with Detail of the pUC Multiple Cloning Sites

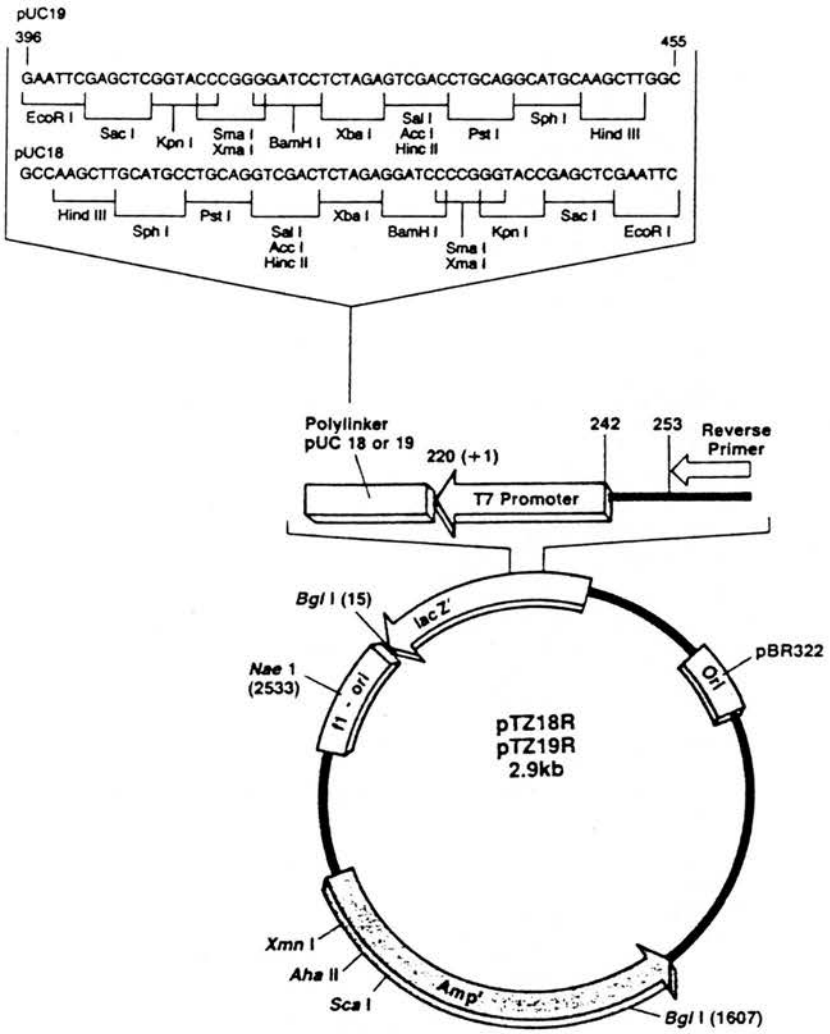


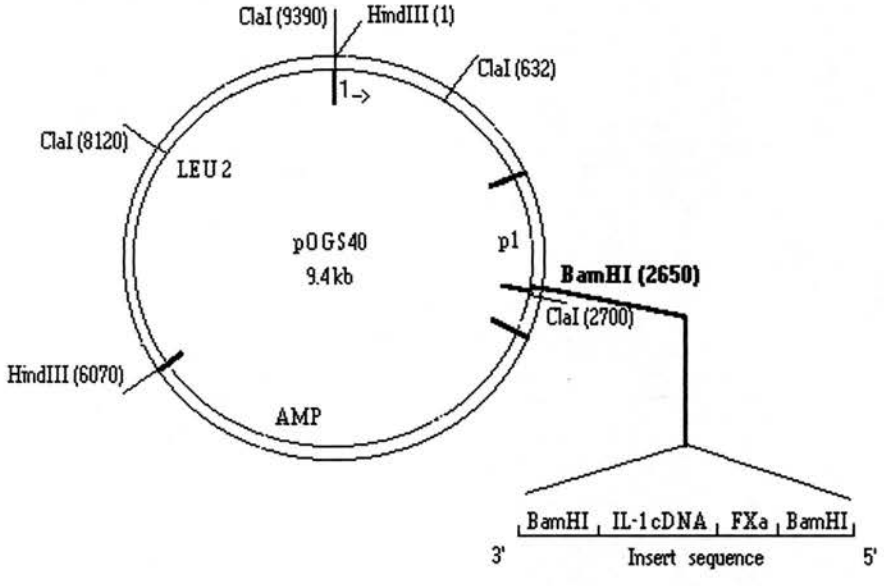
Figure 3.1
The pTZ18R and pTZ19R multifunctional phagemids (Mead et al., 1986) were purchased from Pharmacia. These vectors permit DNA cloning, dideoxy sequencing, *in vitro* mutagenesis and *in vitro* transcription in one system. The vectors contain both the f1 and pBR322 origins of replication for generation of double-stranded DNA or large amounts of single-stranded DNA. If the host cell containing the phagemid is superinfected with the specially designed helper phage M13K07, replication will be initiated at the f1 origin, allowing single-stranded DNA to be produced.

The Multiple Cloning Site (MCS) from pUC18 and pUC19, respectively, has been introduced to facilitate cloning. An ampicillin resistance gene for selection on LB/Amp plates and the *lacZ'* gene for blue/white screening of recombinants on x-gal plates are also carried by the phagemids. The position of the reverse sequencing primer site is indicated.

The phagemids also carry the highly specific bacteriophage T7 promoter immediately adjacent to the multiple cloning site for *in vitro* synthesis of large amounts of specific RNA. This function was not utilised during the study being reported here.

FIGURE 3.2

pOGS40 Expression Vector Cloning



Sequencing primer:

5' CAGGAGAAATCCGAGTG 3'

Figure 3.2
pOGS40 vector is an *E. coli* / yeast shuttle vector used for expressing recombinant protein as a fusion product with the yeast p1 protein. The fusion protein is cleaved by the action of Factor Xa restriction protease to release the recombinant protein.

Only features of the pOGS40 vector which are important with respect to cloning are shown on this diagram. Features which have been incorporated for expression purposes will be detailed in Chapter 4.

A BamHI restriction enzyme site within the 3' end of the p1 gene is used for insertion of cDNA sequences. Insert sequences are flanked at the 5' end by bases encoding a Factor Xa site. BamHI restriction enzyme sites are appended at each end for insertion into the vector.

An ampicillin resistance gene for selection of *E. coli* recombinants on LB/Amp plates and a Leucine module is carried for selection in yeast are also carried. The pOGS40 sequencing primer, derived from the p1 protein sequence (Mellor et al., 1985), binds 61 base pairs upstream of the BamHI insertion site.

In vivo, IL-1α and IL-1β are both initially produced as a proproteins which are enzymatically cleaved to yield the mature proteins. Both proprotein and mature protein coding sequences were to be inserted into pOGS40 and PCR primers were designed accordingly. The start position of sequences encoding the mature proteins was assumed by analogy with bovine and human sequences. Ligation of the resultant PCR products into BamHI digested pOGS40 should yield constructs in which the inserts are in frame for correct protein synthesis.

PCR techniques were employed for cloning both ovine IL-1α and IL-1β cDNAs, although the approaches were slightly different for each. Briefly, the cloning strategy was as follows: Total RNA obtained from LPS stimulated alveolar macrophages was enriched for polyA$^+$ RNA. First strand cDNA synthesised by reverse transcriptase was used as the template for IL-1 cDNA amplification by PCR. Initial PCR primers were derived from published human and/or bovine sequences and secondary primers were derived from the resultant ovine sequences. All PCR products were cloned into pTZ18R and pTZ19R vectors for sequencing and probe generation, ie. a number of overlapping IL-1β sequences (together comprising 32bp 5' leader sequence, an 801bp coding sequence and 145bp of 3' untranslated sequence), and a single IL-1α sequence (consisting of an 807bp coding sequence plus 19bp of the 3' untranslated sequence). The proprotein and mature protein coding sequences for both ovine IL-1α and IL-1β were also cloned into the pOGS40 expression vector.

RESULTS

3.1 Alveolar Macrophages

Alveolar macrophages were obtained by bronchoalveolar lavage at postmortem of gnotobiotic sheep. Careful excision of the lungs ensured minimal blood contamination. Hypotonic lysis removed any red blood cells present. On average $\geq 5x10^8$ macrophages were obtained from a lung lavaged with 2 litres Hanks balanced salt solution. The macrophages, washed and resuspended in Iscoves serum free medium, were allowed to adhere to plastic culture dishes for 16h prior to stimulation with 10μg/ml LPS.

3.2 PolyA$^+$ RNA and cDNA Synthesis

Total RNA extracted from these LPS stimulated macrophages was enriched for polyA$^+$ RNA. Samples from three different animals run on a denaturing agarose gel display the characteristic total RNA pattern with prominent 18s (1.8kb) and 28s (4.4kb) RNA and numerous intermediate bands (Fig. 3.3.a). PolyA$^+$ RNA synthesised from one of these samples appears as a very faint smear on the same gel. (See Sect. 2.3.7 for northern blot analyses of these preparations). On average, 1 x 10^8 macrophages yielded 80μg total RNA of which ≤5% was polyA$^+$ RNA. cDNA was synthesised from polyA+ RNA as template, using an Amersham cDNA synthesis kit with either oligo-$dT_{12\text{-}18}$ or random hexamer primers. The efficiency of first strand cDNA synthesis was 5-10% as estimated by α^{32}P-dATP incorporation and the labelled cDNAs both contained a comprehensive range of sizes with greatest density between 500bp and 2000bp as visualised on agarose gels. An autoradiograph of such a gel is shown in Fig. 3.3.b as an example.

3.3 Cloning Ovine IL-1β cDNA

As ovine IL-1α cDNA was cloned subsequent to IL-1β and the methodology was therefore more refined, ovine IL-1β cDNA cloning will be described first.

3.3.1 Cloning and Sequencing IL-1β cDNA in pTZ18R and pTZ19R Vectors

3.3.1.1 PCR Primers for Amplification of Ovine IL-1β cDNA

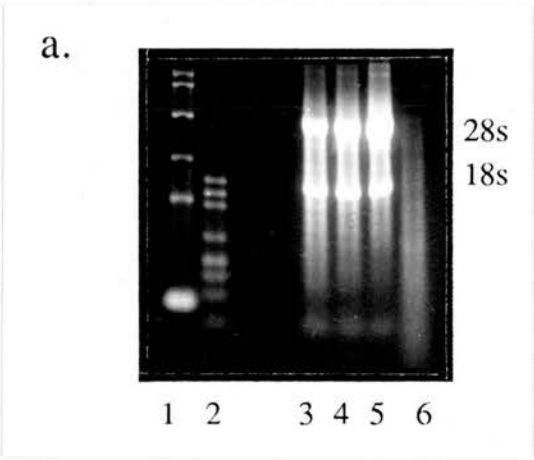
Figure 3.4 is a compilation of all the primers used for amplification of IL-1β cDNA sequences. The derivation of individual primers will be detailed in the following text where appropriate. A diagrammatic representation of the steps involved in IL-1β cloning is shown in Fig 3.5.

FIGURE 3.3

a. Electrophoretic Separation of Total RNA Extracted from Alveolar Macrophages

Figure 3.3.a
RNA from ovine alveolar macrophages stimulated with LPS for 4h was electrophoresed through denaturing agarose gels as per Sections 2.3 and 2.5.1.1. Total RNA samples from three different sheep show the characteristic pattern with prominent 28s and 18s bands as indicated. PolyA$^+$ RNA prepared from one of these samples appears as a faint smear covering the size range 250 - 4400 bases.

Lanes 1 and 2, High and low molecular weight, respectively, RNA markers (see 2.2.2.4 for sizes);
Lane 3, 10µg RNA - Sheep No.1;
Lane 4, 10µg RNA- Sheep No.2;
Lane 5, 10µg RNA - Sheep No.3;
Lane 6, 4µg polyA$^+$ enriched RNA - Sheep No.1.



b. Autoradiograph of cDNA Synthesised from PolyA$^+$ Enriched RNA

Figure 3.3.b
cDNA synthesised from Sheep No.1 polyA$^+$ RNA (shown above) by reverse transcriptase using an Amersham cDNA synthesis kit with added [α-^{32}P]-dCTP was electrophoresed through a 1.5% agarose gel as per Section 2.5.1.2. The dried gel was exposed on Amersham XAR film and shows a range of cDNA sizes with greatest density between 500bp and 2000bp.

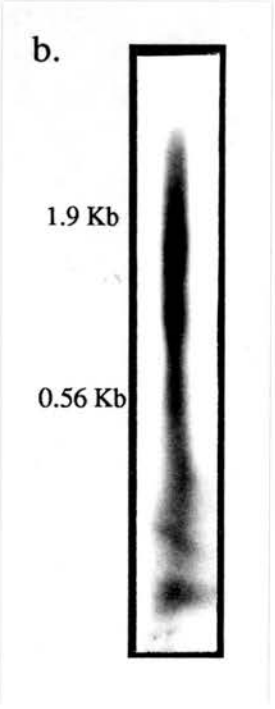


Figure 3.4

B5.1 and B3.1 are mixed primers chosen from consensus sequences in human and bovine IL-1β cDNAs (March et al., 1985; Malisewski et al., 1988). In both bo = A and hu = G. This pair was used for amplifying the central region of ovine IL-1β cDNA. Primers B5.2, B3.2 and B3.3 were chosen from the resultant ovine sequence. Primer B5.3 was selected from the 3' untranslated region of the bovine sequence. Primer dT was used for anchored PCR with polyA tailed cDNA. Clones **β1** - **β3** were sequenced and used for probe generation.

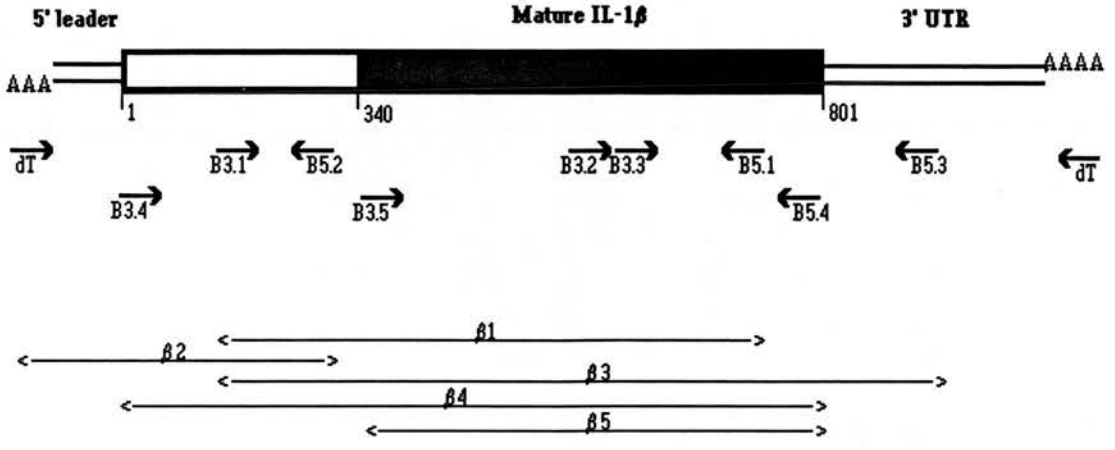
Primers B3.4, B3.5 and B5.4 were designed for use in producing expression vector cassettes. The additional bases at the 5' end of each primer are required for the expression vector constructs. BamHI digestion sites and bases encoding the FXa recognition site are indicated. The amino acids forming the Factor Xa recognition site are shown in small capital letters. PCR products were cloned into both pTZ18R/19R and pOGS40 expression vectors. Clones **β4** and **β5** were excised from pTZ with BamHI and cloned into pOGS40 expression vectors.

IL-1 nucleotide numbers are indicated at each end of IL-1 specific sequences within the primers.
Primers were used at concentrations of 10-20pM.

PCR products which resulted in clones **β1** - **β5** in pTZ18R/19R are shown at the bottom of the diagram.

FIGURE 3.4

PCR Primers used for Ovine IL-1β cDNA Amplification



Sense primers: **5' ----> 3'**

B3.1 171CAACAA$^{A}/_{G}$CTTCAGGCAGG190

B3.2 457GTGGTGTTCTGCATGAGC474

B3.3 498CAACAAGATTCCTGTGGCC516

B3.4 CCCGGGATCCATTGAAGGTAGG 1ATGGCAACCGTACCTGAACCC21

B3.5 CCCGGGATCCATTGAAGGTAGG 340GCAGCCGTGCAGTCAGTAAAATGC363

BamHI — Factor Xa recognition site (I E G R)

Anti-sense primers: **5' ----> 3'**

B5.1 710GTGCTGATGTACCAGTT$^{A}/_{G}$GGG690

B5.2 314GTTTCGAAGATGACAGGC297

B5.3 1011TGGCTCAGTGGATGGGG995

B5.4 CCCGGGATCC 801TTAGGGAGAGAGGGTTTCC783

BamHI

FIGURE 3.5

Scheme of the Steps Involved in Cloning and Sequencing Ovine IL-1β

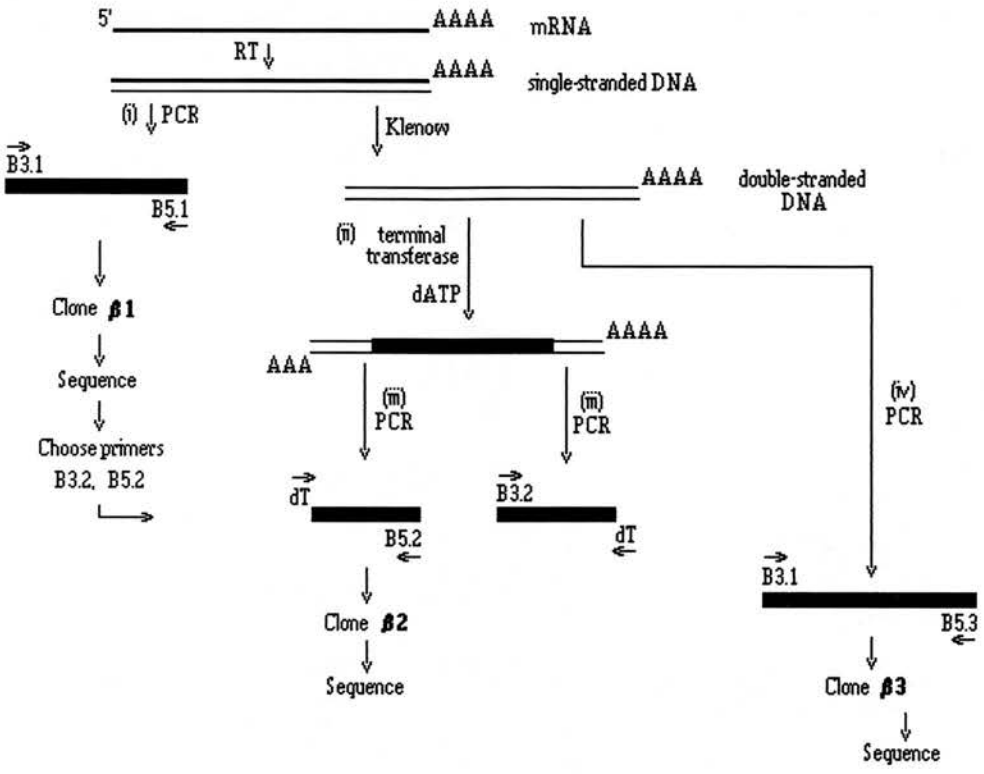


Figure 3.5
Single-stranded cDNA was synthesised from polyA$^+$ RNA by reverse transcriptase (RT) and double-stranded cDNA by the further action of Klenow T7 DNA polymerase. (Amersham cDNA synthesis kit).
The full IL-1β cDNA sequence was obtained via a number of PCR reactions and cloning steps which are represented diagrammatically in this figure. Clone designations are also indicated.

(i) Amplification of a central section of the cDNA with primer pair B3.1/B5.1. This PCR product was cloned into pTZ18R/pTZ19R and sequenced. This is Clone **β1**.

(ii) Tailing the 5' terminal with an oligo-dA sequence.

(iii) Anchored PCRs using specific sequence primers, B3.2 or B5.2, paired with oligo-dT to obtain sequences flanking the central clone. Cloning and sequencing in pTZ18R/19R yielded the 5' flanking sequence, Clone **β2**, but only partial 3' flanking sequence.

(iv) Amplification of the 3' flanking sequence using primer pair B3.1/B5.3. Cloning and sequencing yielded Clone **β3**.

3.3.1.2 PCR amplification of the IL-1β central region – Nucleotides 171-710

PCR primers B5.1 and B3.1, corresponding to nucleotides 171-190 and 710-690 respectively (Fig. 3.4), are mixed primers chosen from consensus sequences in human and bovine IL-1β cDNAs (March et al., 1985, Maliszewski et al., 1988).

This primer pair, B3.1/B5.1, gave a PCR product of the expected size, 535bp, as seen when run on agarose gels (Fig. 3.6.a). Magnesium concentration, which can affect PCR efficiency (Bell, 1989), did not appear to be crucial for IL-1β amplification but Ohara buffer (1.5mM $MgCl_2$; 3mM DTT; 50mM KCl; 100μg/ml BSA; 10mM TrisHCl pH8.8) (Ohara et al., 1989) was marginally better than a commercial Anglian buffer (6.7mM $MgCl_2$; 10mM β-ME; 16.6mM $(NH_4)_2SO_4$; 6.7mM EDTA; 100μg/ml BSA; 67mM TrisHCl pH8.8). Standard reaction parameters were; denaturation 93°C/30sec, primer annealing 50°C/30sec, polymerisation 72°C/2.5min; followed by 72°C/5min to allow full extension of synthesised strands. The amplification was both specific and efficient, giving good yields of IL-1β product, >2μg from 1ng first strand cDNA after 35 cycles.

The PCR band was excised from an agarose gel, purified by adsorption to silica (Geneclean) and ligated into pTZ18R vector. Transformation of *E. coli* JM101 cells with this ligated product yielded a number of recombinant colonies on LB/Amp plates. An agarose gel of EcoRI and HindIII restriction endonuclease digested vector preparations from these colonies shows the presence of different insert sizes. One insert, shown as lane 3 in Fig.3.6.a, appeared to be of the correct size for a dimer. Both uncut and cut preparations of this clone hybridised strongly with the original PCR product (Fig.3.6.b) but uncut pTZ18R and a cDNA preparation from a negative colony which were run on the gel as controls did not hybridise. This positively hybridising clone was called **β1**. All the IL-1 clones generated during this study are listed in Table 3.3 at the end of this Results section. The insert excised from pTZ18R by EcoRI/HindIII digestion was ligated into pTZ19R to place it in the opposite orientation for single strand preparation. Single stranded DNA in both orientations was sequenced using the dideoxy chain termination method with T7 DNA polymerase (Sequenase II, USB Ltd.). This central **β1** clone was 96.8% and 78.2% similar to the equivalent bovine and human sequences respectively.

3.3.1.3 Amplification of sequences flanking β1

3.3.1.3.1 Anchored PCR

Comparison of the leader and 3' untranslated sequences of human, murine and bovine IL-1β showed no obvious region of homology on which PCR primers could be based. Anchored PCR was therefore the next approach for obtaining sequences flanking the **β1** sequence. This method utilises a fixed sequence for

FIGURE 3.6

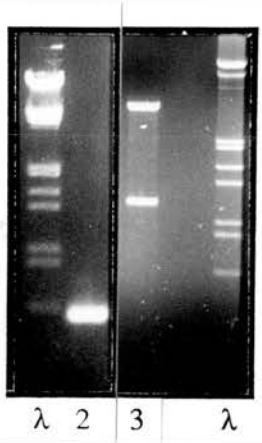
Cloning IL-1β Nucleotides 171 - 710 (Clone β1)

a. cDNA Amplification by PCR using Primer Pair B3.1/B5.1

Single-strand cDNA was amplified with mixed primers chosen from consensus sequences in bovine and human IL-1β cDNAs and the product electrophoresed through 1.5% agarose gels.

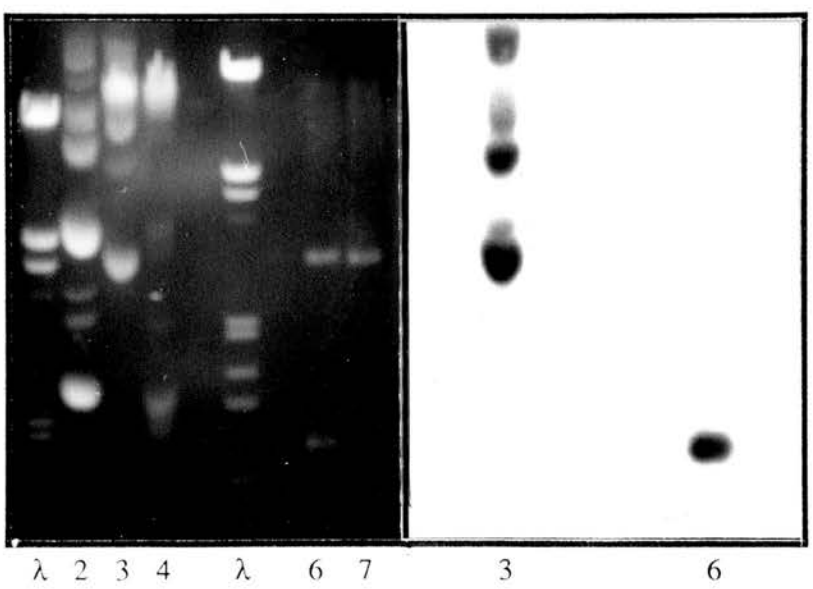
Lane 1, λ markers ; Lane 2, Ovine IL-1β PCR product at 535bp;

Lane 4, pTZ18R clone digested with EcoRI and HindIII to give pTZ18R at 2900bp and insert at 1100bp; Lane 5, λ markers.



λ EcoRI/HindIII markers (21226, 5146, 4973, 4268, 3530, 2027, 1904, 1709, 1375, 947, 831, 564, 125 base pairs)

b. Selection of pTZ18R Clones by EcoRI/HindIII Digestion and Hybridisation with ^{32}P-PCR-Product



Vector preparations from selected JM101 colonies and the EcoRI/HindIII digested preparation seen above were electrophoresed through a 1.5% agarose gel together with the pTZ18R preparation used for the original ligations (left half of the picture). The gel was blotted and hybridised with the original PCR product labelled with ^{32}P (right half of the picture). Undigested samples in lanes 2-4 and digested samples in Lanes 6-7 were electrophoresed as two independent groups on the same gel, each with its own set of λ markers.

Lane 1, λ markers; Lane 2, original uncut pTZ18R; Lane 3, uncut pTZ18R from the positive colony with 1100bp insert; Lane 4, uncut pTZ18R from a negative colony; Lane 5, λ markers; Lane 6, EcoRI/HindIII digest of positive sample with 1000bp insert; Lane 7, EcoRI/HindIII digest of pTZ18R from the negative colony.

one primer together with a specific sequence for the second primer. The polyA tail at the 3' end of the cDNA could be utilised as one such anchored primer site. cDNA specifically primed with the PCR primer, B3.1, was tailed with a stretch of 20 dA nucleotides, using the terminal transferase reaction, which provided a similar fixed sequence at the 5' terminus. Primers B3.1 and B5.1 were paired with oligo-dT as the anchored primer and should have yielded the outstanding sequences at either end of the cDNA. The annealing temperature was lowered to 42°C because of the lower Tm of the oligo-dT primer but other PCR parameters remained unchanged. Initial PCRs using either dT_{12-18} or dT_{19-24} as the anchored primer produced smears which hybridised fully with ^{32}P labelled **β1** as shown by a 3 hour exposure of dT_{19-24} Southern blots (Fig. 3.7a). A simultaneous control reaction with B3.1/B5.1 primers confirmed both the specificity of these two primers and the intactness of the tailed cDNA. Although it seemed most likely that the oligo-dT was mispriming, further PCRs were carried out using nested primers, B5.2 and B3.2 selected from the **β1** clone sequence, in an attempt to make the reaction more specific. Control PCRs using these primers yielded products of the expected sizes but anchored PCRs again resulted in smears (Fig. 3.7b). Various buffers were tried, with or without the addition of DMSO to relax secondary structure, the most successful of which was the low magnesium Ohara buffer with no added DMSO. Three sequential reaction sets using nested primers and increasing annealing temperatures (42°C, 50°C, 55°C), resulted in somewhat tighter smears (Fig 3.7c).

The expected size of the 5' end product (primer pair dT/B5.2) was about 400bp, ie. base pairs 1 to 315 of coding sequence plus <100bp leader sequence (bovine=75bp; human=90bp). Smears covering 200–800bp for the 5' end were therefore excised from agarose gels, purified and cloned into pTZ18R. The largest 5' clones obtained, 694bp, were found to be dimers. These clones yielded the outstanding 145 bases to the start codon plus 32 bases of leader sequence. The cloned sequence -32 to 315 was called **β2.1**. A second 5' end smear, from a different PCR reaction with the same primers, yielded clones which confirmed the sequence apart from T/C differences at nucleotide positions 84 and 191. Four clones were sequenced and found to be identical - clone **β2.2**.

The 3' end product (primer pair dT/B3.2) was expected to be about 1300bp, ie. base pairs 438–801 of coding sequence, plus ≤900bp of 3' untranslated sequence (bovine=876bp; human=616bp). Smears covering 800–2500bp were excised from gels and cloned into pTZ18R. None of the 3' end clones contained full length inserts, the largest size of positively hybridising insert being 600bp. Sequencing of this clone revealed 223 bases of clear IL-1 sequence followed by a 72 base region of compression, ie. bands in all four lanes, followed by a 17

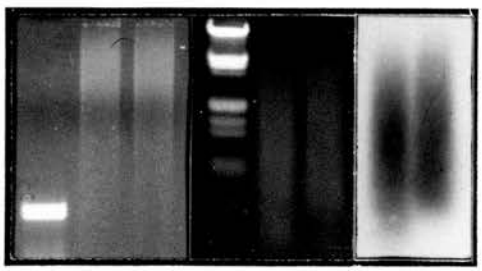
FIGURE 3.7

PCR Reactions using Oligo-dT as the Anchored Primer

a. Use of Oligo-dT_{12-18} vs Oligo-dT_{19-24} Primers

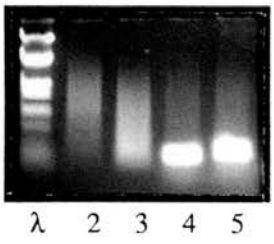
PolyA tailed cDNA was assessed by amplification with theB3.1/B5.1 primer pair. cDNA and polyA-tailed cDNA were used as templates for anchored PCR reactions. Products from B3.1 and B5.1 primers paired with either oligo-dT_{12-18} or oligo-dT_{19-24} were electrophoresed through 1.5% agarose gels. Southern blots of oligo-dT_{19-26} products were hybridised with ^{32}P-**β1**.

Lane 1, Tailed cDNA amplified with B3.1/B5.1 primers; Lane 2, B5.1/dT_{12-18}; Lane 3, B3.1/dT_{12-18}; Lane 4, λ markers; Lanes 5a and 5b, B3.1/dT_{19-24}, unhybridised and hybridised resp.; Lanes 6a and 6b, B5.1/dT_{19-24}, unhybridised and hybridised resp.



b. Use of Nested IL-1β Primers Paired with Oligo-dT

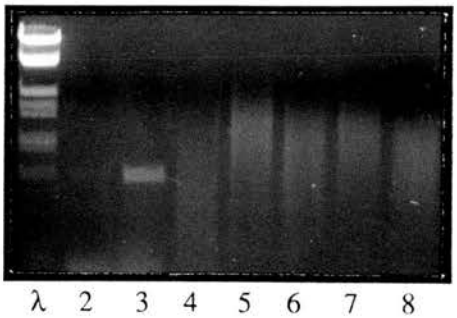
Internal primers B3.2 and B5.2, chosen from the ovine **β1** clone sequence, were paired with oligo-dT_{19-24}.
Lane 1, λ markers; Lane 2, B5.2/dT; Lane 3, B3.2/dT; Lane 4, B5.2/B3.1; Lane 5, B3.2/B5.1.



c. Final PCR Products from Sequential Reaction Sets

Sequential reaction sets were carried out using B3.x and B5.x nested primers paired with oligo-dT. Annealing temperatures were increased with each set of PCR cycles (42°C, 50°C, 55°C). Products electrophoresed through 1.5% agarose gels are shown.

Lane 1, λ markers; Lane 3, control B3.1/B5.1 product;
Lanes 5 and 6, 5' flanking region;
Lanes 7 and 8, 3' flanking region.



base sequence consisting of 14C and 3G residues. The final 17 bases before the vector sequence showed 71% homology with the bovine 785-799 nucleotide sequence. Secondary structure due to a fairly high GC rich sequence was suspected as being the most likely cause of the compressions. Support was lent to this idea by a computer model of the bovine sequence from nucleotides 600-801, which showed a hairpin and potentially awkward secondary structure in this region (Fig. 3.8).

FIGURE 3.8

Potential Secondary Structure of Bovine IL-1β from Nucleotides 600 to 801

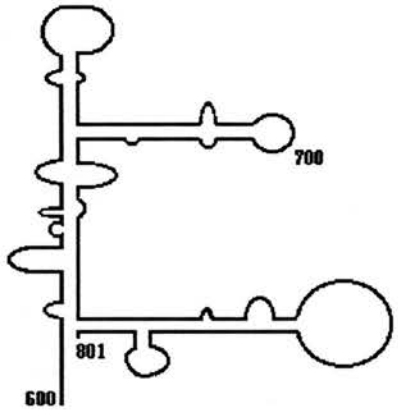


Figure 3.8 The opitmal secondary structure of bovine IL-1β from bases 600 - 801 (Maliszewski et al., 1988) was determined using the method of Freier et al., 1986. The stability of the structure is ΔG_f = -41.8kcal/mol.

3.3.1.3.2 Sequencing 3' cDNA clones – Variations involving destabilisation of secondary structure

A number of sequencing variations designed to destabilise GC bonding and hairpin loops were used in an attempt to sequence through the compression: (i) using deaza dGTP or dITP in place of dGTP; (ii) adding DMSO up to 10%; (iii) varying DNA:primer ratios; (iv) sequencing with *Taq* polymerase at 72°C instead of T7 DNA polymerase at 37°C; (v) sequencing using primer B3.3 and 3 dNTPs only plus a 30 fold dilution of G mix, again to destabilise GC bonding; (vi) adding the single strand DNA binding protein, T32, during the sequencing reaction with deaza dGTP; (vii) running sequencing gels containing 8.3M urea instead of the normal 7.67M urea. None of these methods yielded acceptable sequence off either strand. None of the clones containing smaller inserts yielded any additional sequence.

3.3.1.3.3 λgt10 cDNA library screening

In order to obtain the remaining nucleotides at the 3' end of the IL-1β cDNA sequence the two alternatives considered were either to screen a cDNA library in λgt10 vector or to chose another PCR primer. Screening of λgt10 libraries constructed from hexamer primed cDNA, provided a large number of positively hybridising clones but many inserts were difficult to excise from the vector (Fig. 3.9.a). None of the 3' inserts which could be excised provided any additional sequence (after having been subcloned into pTZ18R). The use of λgt10 primers to PCR across the insert from vector sites again produced some non-specific priming presumably because the vector sequences flanking the insert site and hence the primers, are very AT rich. Without amplification of the insert, direct sequencing was not a viable alternative due to the relative sizes of vector and insert ie. 43.3 and 1.5 kilobases respectively.

A library constructed from the B3.1 primed cDNA used for 5' anchored PCR (see Sect. 3.3.1.3.1) was screened simultaneously but the inserts could not be excised from positive constructs (Fig. 3.9.b). Clone **β1** which was used for probing the Southern blots of digested clones does not cross-hybridise with λgt10. All positive signals therefore correspond to specific inserts.

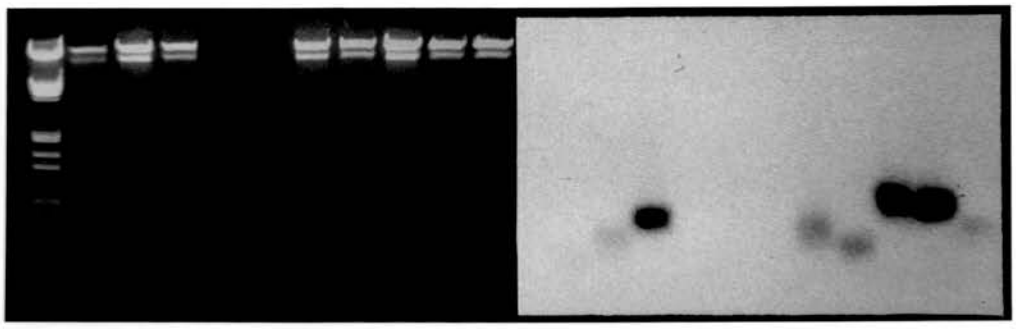
3.3.1.4 PCR amplification of sequence extending into the 3' untranslated region – Nucleotides 171–1011

The most important sequence to obtain, as far as I was concerned, was from bases 1–801, the coding region of the IL-1β cDNA, because information on regulatory sequences within the 3' untranslated sequence was not going to be utilised in the subsequent expression work. One final primer, B5.3 (see Fig. 3.4), was selected from the bovine 3' untranslated region. Even though this region is not well conserved across other species, extremely close ovine:bovine homology had been found in sequences already analysed and it was therefore hoped that the 3' untranslated sequences might not be too divergent. The sequence closest to the stop codon which fulfilled the requirements for a primer consisted of bases 995-1011, the region between 801 and 994 being too GC rich. PCR using B3.1/B5.3 primers produced a product of the expected size, 841bp which hybridised strongly with clone **β1** (Fig. 3.10). Sequencing of the cloned product, **β3**, yielded the outstanding 78 bases between the central **β1** sequence and the stop codon, plus 145 bases of 3' untranslated sequence. The latter did in fact show 93% homology with the bovine IL-1β sequence. The sequence also shows a region from bases 716 - 728 which could have accounted for mispriming by the oligo dT primer and may have been the cause of the compression region.

FIGURE 3.9

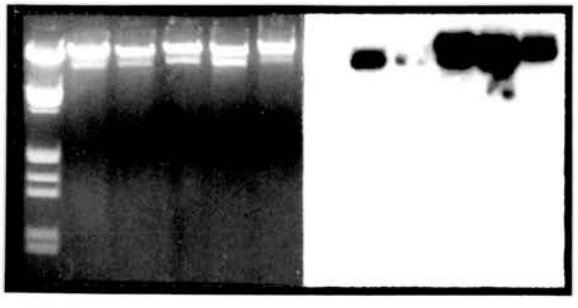
λgt10 / IL-1β cDNA Clones

a. Positive Clones from a Hexamer Primed cDNA Library in λgt10 Vector



The λgt10 library was screened with ^{32}P-**β1**. Clones positive after tertiary screening were digested with EcoRI. 1.5% agarose gels show the range of insert sizes obtained (Lanes 2 - 4 and 7 - 11). Hybridisation of the Southern blotted gel, hybridised with clone **β1**, indicates the presence of IL-1β specific sequences. λ markers are shown as Lane 1. Lanes 5 and 6 contain no sample.

b. Positive Clones from the B3.1 Primed cDNA Library in λgt10 Vector



DNA digested, electrophoresed and blotted as above but hybridisation shows that inserts did not excise from the vector on EcoRI digestion.

FIGURE 3.10

PCR Amplification of IL-1β Nucleotides 171 - 1011 using Primer Pair B3.1/B5.3

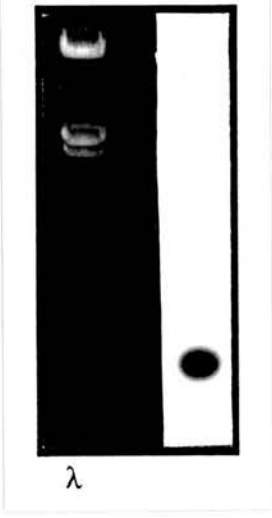


Figure 3.10
cDNA amplification yielded a clean PCR product at the expected molecular weight (841bp), which hybridised with clone **β1**.

Lane 1, λ markers; Lane 2, PCR product on 1.5% agarose gel; Lane 3, Southern blotted gel hybridised with **β1**.

At least 4 clones of each product were fully sequenced in both directions using PCR primers as intermediate sequencing primers. The clones encoding the ovine IL-1β sequence constituted 32bp of 5' leader sequence an 801bp coding sequence and 145bp of 3' untranslated sequence having two potential C/T polymorphisms, at positions 84 and 193, the latter giving A/V on translation. Further potential polymorphisms became apparent during the cloning of expression constructs (see below) and the ovine IL-1β cDNA sequence and its translation are therefore shown in Fig. 3.12 on pg.98.

3.3.2 Production of IL-1β pOGS40 Expression Constructs

From the IL-1β coding sequence obtained above, PCR primers B3.4, B3.4 and B5.4 (see Fig. 3.4) were designed which incorporated sequences required for insertion into the yeast expression vector pOGS40 (see Fig. 3.2). The start position of the mature IL-1β region was assumed from analogy with published sequences of the human and bovine IL-1 proteins (March et al., 1985; Maliszewski et al., 1988). Both the complete sequence coding for the IL-1β proprotein (**TYβp**), residues 1-801, and that coding for the IL-1β processed mature protein (**TYβm**), residues 340-801, were obtained (Fig. 3.11).

The outline of the protocol followed for production of expression constructs was

(i) PCR as above;
(ii) ligation of PCR product into pTZ18R/19R for confirmatory single stranded sequencing;
(iii) excision of insert from pTZ18R with BamHI, gel purification of insert and ligation into pOGS40 vector;
(vi) selection of expression constructs.

The clones in pTZ18R are **β4**, IL-1β proprotein sequence, and **β5**, mature IL-1β sequence (all the IL-1 clones generated are collated in Table 3.3). All **β4** clones sequenced were identical, as were all **β5** clones. However, the **β4** sequence revealed a further four potential polymorphisms with respect to the sequence already obtained, ie. at positions 24, 41, 75, and 163. Differences at positions 41 and 163 lead to alterations on translation ie. C/Y and K/Q respectively. Table 3.1 summarises the sequence differences found, all of which are in the region encoding the N terminal portion of the proprotein. The clone **β4**, which was inserted into the expression vector, and the resultant amino acid incorporated into the recombinant IL-1β proprotein are shown in bold print.

All cDNA preparations were derived from the same polyA$^+$ RNA sample. **β1**, **β2** (ie. β2.1 and β2.1) and **β4** clones were derived from three different cDNA template preparations. **β1** and **β4** were each derived from single PCR reactions. **β2.1** was derived from a set of three sequential PCR reactions and **β2.2** from an independent reaction set.

FIGURE 3.11

PCR Amplification of IL-1β Nucleotides 1 - 801 and 340 - 801 with Primer Pairs B3.4/B5.4 and B3.5/B5.4 Respectively

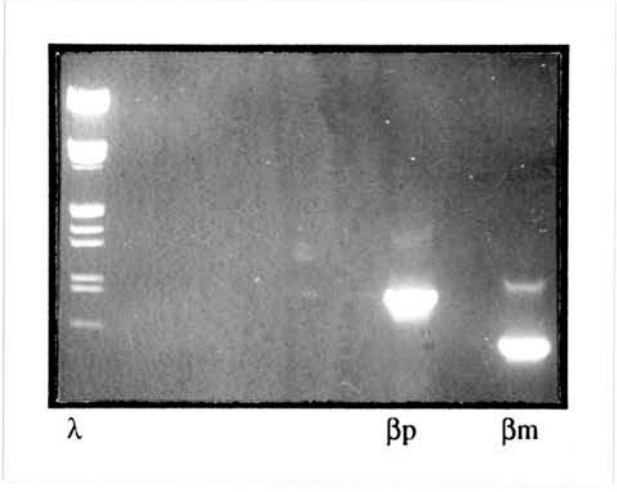


Figure 3.11
cDNA sequences encoding ovine IL-1β proprotein and mature protein were amplified with primers designed to include sequences required for subsequent insertion into expression vectors (see Fig. 3.4). The start of the mature protein was assumed from human (March et al., 1985) and bovine (Maliszewski et al., 1988) IL-1β cDNAs. PCR products on 1.5% agarose gels show bands at the expected sizes, 801 and 462 base pairs respectively.

λ = markers; βp = cDNA for IL-1β proprotein; βm = cDNA for IL-1β mature protein.

TABLE 3.1

Potential IL-1β Polymorphisms Detected in PCR Derived cDNA Clones

Base No.	Clone name	Nucleic Acid		Amino Acid
24	β2.1, β2.2	C	(b)	I
	β4	**T**	(o)	**I**
41	β2.1, β2.2	G		C
	β4	**A**	(b,h,o)	**Y**
75	β2.1, β2.2	T	(b,h,o)	V
	β4	C		**V**
84	β2.1	T	(h)	P
	β2.2, **β4**	**C**	(b,o)	**P**
163	β2.1, β2.2	A		K
	β4	**C**	(b,h,o)	**Q**
191	β1.1, β2.1	C	(h)	A
	β2.2, **β4**	**T**	(b,o)	**V**

Table 3.1
The clone **β4**, which was inserted into the expression vector, and the resultant amino acid incorporated into the recombinant IL-1β proprotein are shown in bold print. Letters in brackets denote the nucleotide present in human (h; March et al., 1985) and bovine (b; Maliszewski et al., 1988) IL-1β cDNA sequences and an ovine sequence published subsequent to this work (o; Andrews et al., 1992).

FIGURE 3.12

Nucleotide and Predicted Amino Acid Sequence of Ovine IL-1β

```
                                                GAACCTTCATTGCCCAGGTTTCTGAAACAGCC  -1

ATGGCAACCGTACCTGAACCCATTAATGAAGTGATGGCTTACTACAGTGATGAGAATGAG  60
M  A  T  V  P  E  P  I  N  E  V  M  A  Y  Y  S  D  E  N  E    20

CTGTTATTTGAGGTCGATGGCCCCAAACAGATGAAGAGCTGCACCCAACACCTGGACCTC 120
L  L  F  E  V  D  G  P  K  Q  M  K  S  C  T  Q  H  L  D  L    40

GGCTCCATGGGAGATGGAAACATCCAGCTGCAGATTTCTCACCAGCTCTACAACAAAAGC 180
G  S  M  G  D  G  N  I  Q  L  Q  I  S  H  Q  L  Y  N  K  S    60

TTCAGGCAGGTAGTGTCGGTCATCGTGGCCATGGAGAAGCTGAGGAGCCGTGCCTACGAA 240
F  R  Q  V  V  S  V  I  V  A  M  E  K  L  R  S  R  A  Y  E    80

CATGTCTTCCGTGATGATGACCTGAGGAGCATCCTTTCATTCATCTTCGAAGAAGAGCCT 300
H  V  F  R  D  D  D  L  R  S  I  L  S  F  I  F  E  E  E  P   100
                                    V
GTCATCTTCGAAACATCCTCCGATGAGCTTCTGTGTGATGCAGCCGTGCAGTCAGTAAAA 360
V  I  F  E  T  S  S  D  E  L  L  C  D  A  A  V  Q  S  V  K   120

TGCAAACTCCAGGACAGAGAGCAAAAATCCCTGGTGCTGGATAGCCCATGTGTGCTGAAG 420
C  K  L  Q  D  R  E  Q  K  S  L  V  L  D  S  P  C  V  L  K   140

GCTCTCCACCTCCTCTCACAGGAAATGAGCCGAGAAGTGGTGTTCTGCATGAGCTTCGTA 480
A  L  H  L  L  S  Q  E  M  S  R  E  V  V  F  C  M  S  F  V   160

CAAGGAGAGGAAAGAGACAACAAGATTCCTGTGGCCTTGGGTATCAGGGACAAGAATCTA 540
Q  G  E  E  R  D  N  K  I  P  V  A  L  G  I  R  D  K  N  L   180

TACCTGTCTTGTGTGAAAAAAGGTGATACACCGACCCTGCAGCTGGAGGAAGTAGACCCC 600
Y  L  S  C  V  K  K  G  D  T  P  T  L  Q  L  E  E  V  D  P   200

AAAGTCTACCCCAAGAGGAATATGGAAAAGCGATTCGTCTTCTACAAGACAGAAATCAAG 660
K  V  Y  P  K  R  N  M  E  K  R  F  V  F  Y  K  T  E  I  K   220

AACACAGTTGAATTTGAGTCTGTCCTGTACCCTAACTGGTACATCAGCACTTCTCAAATC 720
N  T  V  E  F  E  S  V  L  Y  P  N  W  Y  I  S  T  S  Q  I   240

GAAGAAAAGCCCGTCTTCCTGGGACGTTTTAGAGGTGGCCAGGATATAACTGACTTCAGA 780
E  E  K  P  V  F  L  G  R  F  R  G  G  Q  D  I  T  D  F  R   260

ATGGAAACCCTCTCTCCCTAAAGAAGCCATACGCAGGGGTCCACGTGGGCTGAATAACCCCGAG 844
M  E  T  L  S  P  *                                          266

GACTGGCAGAAGGGAAGGGAAGAACACCAGCCGCAGCCTGAACCTCACTGTTGTGTGATCCATGCC 910

CAACTGCCTCCCCTGTATTCGTGCTGAGACGCTCTC                         946
```

Figure 3.12
cDNA and protein sequences are numbered starting with the initiator Met. The presumed start of the mature protein, Ala-114, is designated **V**. Potentially polymorphic residues, detected from sequencing a number of overlapping clones derived from various PCR amplifications (see Table 3.1), are shown in bold print. The sequence shown is that of Clone **β4** which was used in expression studies.

Although inserts cut out of pTZ18R with BamHI were gel purified twice on 1.2% agarose gels before being inserted into BamHI cut pOGS40 vector, minimal amounts of pTZ18R were always carried over into the ligation mix. Because the insert ligates more efficiently into pTZ18R than into pOGS40 and transfection with the smaller vector is also more efficient, transfected *E. coli* JM83 colonies positive for both IL-1β and pOGS40 were selected by colony hybridisation on duplicate filters following the method of Buluwela et al., 1989. pOGS40 was identified by probing with the Leu2 fragment (bp 6070-9390 excised from the vector with HindIII), which does not cross-hybridise with pTZ. The orientation of the insert in pOGS40 constructs was established by digestion with ClaI and BalI restriction enzymes, the latter providing a unique assymmetric site in the insert. Expected fragment sizes are shown in Table 3.2.

Double stranded sequencing of selected clones, using a p1 sequence specific primer (see Fig. 3.2) situated 61 base pairs upstream of the BamHI insertion site, confirmed that the cDNA insert was in frame for correct protein synthesis. pOGS40 clones are designated **TYβp** for the proprotein coding region and **TYβm** for the mature coding region.

TABLE 3.2

Orientation of IL-1β cDNA Inserts in pOGS40 Constructs as Determined by ClaI/BalI Restriction Endonuclease Digestion

		Correct	Incorrect
a. **TYβp**		5420	5420
		2260	**2061**
		1270	1270
		632	632
		515	515
		94	**293**
	Total	10191bp	10191bp
b. **TYβm**		5420	5420
		2436	**2062**
		1270	1270
		632	632
		94	**468**
	Total	9852bp	9852bp

Table 3.2
pOGS40/IL-1 cDNA preparations were digested with ClaI and BalI restriction endonucleases. pOGS40 contains four ClaI sites (see Fig. 3.2). The presence of a single asymmetrical BalI site in the IL-1β sequence leads to the fragment sizes shown in bold print, as determined by the orientation of IL-1 cDNA in the vector.

3.4 Cloning and Sequencing Ovine IL-1α cDNA

From the experience gained in cloning IL-1β, a much simpler strategy was employed for cloning IL-1α. Broadly, the entire ovine IL-1α cDNA coding sequence was amplified directly using primers with appended pOGS40 insertion sequences. The resultant PCR product was inserted independently into pTZ18R/19R and pOGS40 vectors.

3.4.1 PCR Amplification of IL-1α cDNA – Nucleotides 1–826 and 358–826

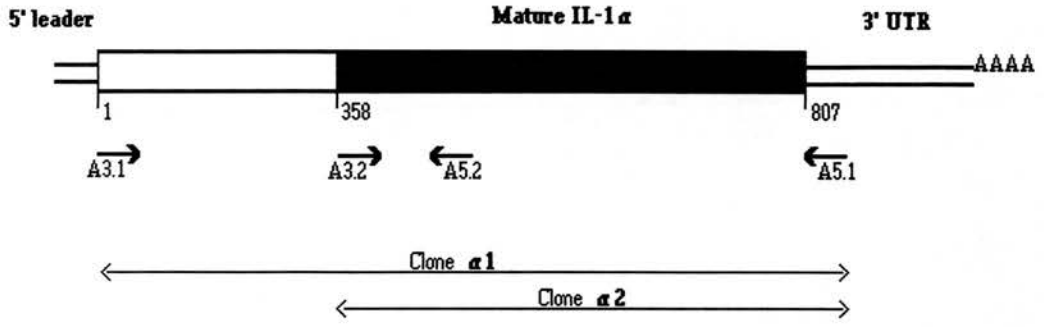
In view of the high degree of homology exhibited by ovine and bovine IL-1β cDNAs, primers were chosen from the bovine sequence (Maliszewski et al., 1988) which would cover the complete coding sequence for the IL-1α proprotein (**TYαp**), residues 1-807. The 3' antisense primer of necessity extended into the 3' untranslated sequence because the sequence leading up to the stop codon contains no stretches showing a GC content suitable for inclusion into a primer. From the sequence of the resultant PCR product, a specific ovine PCR primer was chosen for amplification of the IL-1α mature protein (**TYαm**), residues 358-807. The amino terminus of the mature protein was assumed from the bovine sequence. Sequences for insertion into the pOGS40 expression vector, plus additional bases to protect the BamHI restriction site, were also included in the primers (Fig. 3.13).

PCR amplification parameters were 35 cycles of 93°C/30s denaturation; 56°C/2.5min annealing; 72°C/30sec polymerisation followed by a final strand extension at 72°C for 5min. Addition of 5% DMSO to Ohara buffer (1.5mM $MgCl_2$) increased IL-1αp yields but did not dramatically affect IL-1αm yields (Fig. 3.14). No product was obtained with Anglian buffer (6.7mM $MgCl_2$) either with or without added DMSO. The expected product sizes were 826bp and 479bp for pro- and mature IL-1α respectively. Faint ghost bands of obviously incorrect sizes were ignored. The PCR products were extracted with phenol/chloroform, end-filled and phosphorylated, then purified on preparative 0.8% low gelling temperature agarose gels. The bands were excised and twice re-extracted with phenol/chloroform. 600ng of each was sequenced directly by double stranded sequencing using the PCR primers and confirmed as being IL-1α.

These PCR products were used for ligation into pTZ18R/19R vectors for sequencing purposes, as well as for direct ligation into the pOGS40 expression vector.

FIGURE 3.13

Primers Used for Ovine IL-1α cDNA Cloning



Sense primers: 5' ----> 3'

A3.1 CCCGGGATCCATTGAAGGTAGG 1ATGGCCAAAGTCCCTGACC

A3.2 ATACCCGGGATCCATTGAAGGTAGG

358AGTAACGTGAAATACAAC375

BamHI — Factor Xa recognition site (I E G R)

Anti-sense primers: 5' ----> 3'

A5.1 ATACCCGGGATCC 826GTAGAGTGCACAGTCAAGGC807

BamHI

A5.2 410TTCAGGTAGCATTCCTGG393

Figure 3.13
Primers A3.1, A3.2 and A5.1 were designed for use in expression vector cassettes. A3.1 and A5.1 sequences were chosen from the bovine IL-1α cDNA sequence (Maliszewski et al., 1988) and A3.2 was chosen from the resultant ovine sequence. IL-1 nucleotide numbers are indicated for each primer. Nucleotides for insertion into pOGS40 expression vector were appended. BamHI restriction endonuclease sites and the Factor Xa recognition site are indicated. Additional 5' bases were added to protect the BamHI digestion site. PCR products from primer pairs A3.1/A5.1 and A3.2/A5.2 were cloned and sequenced in pTZ18R/19R. Clones **α1** and **α2** respectively.

Primer A5.2 was derived from the cloned ovine sequences and used as an internal sequencing primer.

Primers were used at 10-20pM.

FIGURE 3.14

PCR Amplification of Ovine IL-1α Proprotein and Mature Protein Coding Sequences - Effects of Mg^{2+} Concentration and DMSO

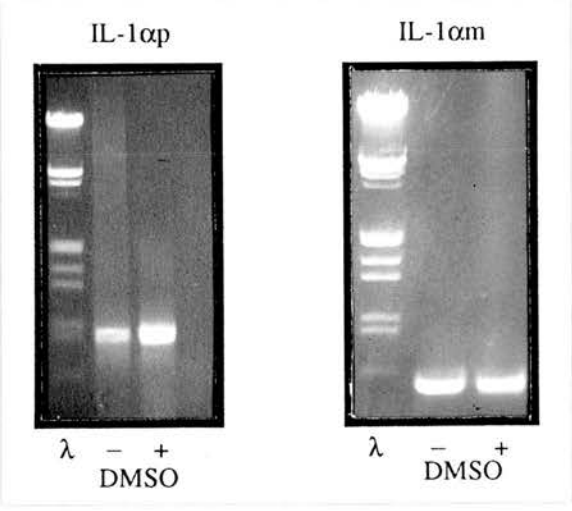


Figure 3.14
IL-1α proprotein and mature protein coding sequences were amplified by PCR using primer pairs A3.1/A5.1 and A3.2/A5.1 respectively. PCR products electrophoresed on 1.5% agarose gels show the effect of adding 5% DMSO to the buffer. PCR products were obtained with Ohara buffer (1.5mM $MgCl_2$), but no product was obtained with Anglian buffer (6.7mM $MgCl_2$) as shown in Lane 4 on the IL-1αp gel.

3.4.2 Cloning and Sequencing IL-1α PCR Products in pTZ18R/19R Vectors

100ng of filled in PCR product was blunt-end ligated into SmaI cut pTZ18R then pTZ19R vectors and single stranded DNA synthesised and sequenced. For obtaining full sequence in both directions an antisense sequencing primer, A3.3, was chosen from the ovine sequence as shown in Fig 3.13. No sequence differences were seen either between individual IL-1α**1** (proprotein) and IL-1α**2** (mature protein) clones or their primary PCR products. Fig. 3.15 shows the ovine IL-1α cDNA sequence and its amino acid translation.

3.4.3 Cloning IL-1α into pOGS40 Expression Vector

Filled in PCR products were cut with BamHI restriction endonuclease and phosphorylated prior to low gelling temperature (lgt) agarose gel purification and ligation into CIP treated BamHI cut pOGS40 vector at a ratio of 300ng insert to 500ng pOGS40. Numerous colonies of transformed *E. coli* JM83 bacteria were obtained, all of which contained positive inserts. BamHI digestion confirmed insert size but as no suitable enzyme sites were available for orientation digestion, vectors containing correct inserts were determined by double stranded sequencing using the p1 specific pOGS primer (see Fig. 3.2).

FIGURE 3.15

Nucleotide and Predicted Amino Acid Sequence of Ovine IL-1α

```
atggccaaagtccctgacctctttgaagacctgaagaactgttacagtgaaaatgaagac    60
M  A  K  V  P  D  L  F  E  D  L  K  N  C  Y  S  E  N  E  D      20

tacagttctgaaattgaccacctctctctgaatcagaagtccttctatgatgcaagctat   120
Y  S  S  E  I  D  H  L  S  L  N  Q  K  S  F  Y  D  A  S  Y      40

gagccacttcgtgaggaccacatgaataagtttatgtccctggatacctcggaaacctcc   180
E  P  L  R  E  D  H  M  N  K  F  M  S  L  D  T  S  E  T  S      60

aagacatccaggcttagcttcaaggagaatgtggtgatggtgacagccaatggcaagatt   240
K  T  S  R  L  S  F  K  E  N  V  V  M  V  T  A  N  G  K  I      80

ctgaagaagagacggttgagtttaaatcagttcatcaccgatgatgacctggaagccatt   300
L  K  K  R  R  L  S  L  N  Q  F  I  T  D  D  D  L  E  A  I     100
                                                   V
gccaatgataccgaagaagaaatcatcaagcccagatcagcacattacagcttccagagt   360
A  N  D  T  E  E  E  I  I  K  P  R  S  A  H  Y  S  F  Q  S     120

aacgtgaaatacaactttatgagagtcatccaccaggaatgcatcctgaacgacgccctc   420
N  V  K  Y  N  F  M  R  V  I  H  Q  E  C  I  L  N  D  A  L     140

aatcaaagtataattcgagatatgtcaggtccatacatgacggctgctacattaaataat   480
N  Q  S  I  I  R  D  M  S  G  P  Y  M  T  A  A  T  L  N  N     160

ctggaggaggcagtgaaatttgacatggttgcttatgtatcagaagaggattctcagctt   540
L  E  E  A  V  K  F  D  M  V  A  Y  V  S  E  E  D  S  Q  L     180

cctgtgactctaagaatctcaaaaactcaactgtttgtgagtgctcaaaatgaagacgaa   600
P  V  T  L  R  I  S  K  T  Q  L  F  V  S  A  Q  N  E  D  E     200

cccgtcttgcataaggagatgcctgagacacccaaaatcatcaaagatgagaccaatctc   660
P  V  L  H  K  E  M  P  E  T  P  K  I  I  K  D  E  T  N  L     220

ctcttcttctgggaaaagcatgggtctatggactacttcaaatcagttgcccatccaaag   720
L  F  F  W  E  K  H  G  S  M  D  Y  F  K  S  V  A  H  P  K     240

ttgttcattgccacaaagcaagaaaaactggtgcacatggcaagcgggccgccctcgatc   780
L  F  I  A  T  K  Q  E  K  L  V  H  M  A  S  G  P  P  S  I     260

actgactttctgatattggaaaaatagccttgactgtgcactctac                  826
T  D  F  L  I  L  E  K  *                                       280
```

Figure 3.15
DNA and protein sequences are numbered starting from the initiator Met. The presumed start of the mature protein, Ser-120, is designated **V**.

3.5 Summary of Ovine IL-1 Clones

The individual ovine IL-1 clones generated are summarised in Table 3.3.

TABLE 3.3

Ovine IL-1 Clone Nomenclature

Clone	PCR primers	IL-1 base Nos	Insert size bp	Vector
IL-1β1	B3.1/B5.1	171 - 710	540	pTZ18R/19R
IL-1β2.1	dT/B5.2	-32 - 315	347	"
IL-1β2.2	dT/B5.2	-32 - 315	347	"
IL-1β3	B3.1/B5.3	171 - 1011	841	"
IL-1β4	B3.4/B5.4	1 - 801	801	"
IL-1β5	B3.5/B5.4	340 - 801	462	"
IL-1.TYβp	B3.4/B5.4	1 - 801	831	pOGS40
IL-1.TYβm	B3.5/B5.4	340 - 801	492	"
IL-1α1	A3.1/A5.1	1 - 826	864	pTZ18R/19R
IL-1α2	A3.2/A5.1	358 - 826	504	"
IL-1.TYαp	A3.1/A5.1	1 - 826	851	pOGS40
IL-1.TYαm	A3.2/A5.1	358 - 826	491	"

Table 3.3

IL-1 fragment sizes differ from the vector insert sizes where the latter include pOGS40 insertion nucleotides.

3.6 Interleukin-1 Sequence Comparisons

The ovine IL-1α PCR product and all clones sequenced yielded identical sequences for the cDNA which consisted of 807bp of coding sequence plus 19bp 3' untranslated sequence. IL-1β sequence consisted of 32bp leader, 801bp coding sequence and 145bp of the 3' untranslated sequence. 6 nucleotide differences were obtained from the various IL-1β clones sequenced. All clones from any one PCR reaction yield consistent sequences. There is only 25% identity between ovine IL-1α and IL-1β. Similar identities have been found between alpha and beta forms of bovine, human and murine IL-1s. Table 3.4 shows the degree of cross species homology exhibited by the ovine IL-1s. The IL-1α and IL-1β sequence comparisons from which these figures were derived are shown in Figs. 3.16 and 3.17 respectively. The start of the mature proteins are denoted ▼, potential glycosylation sites are underlined and potentially polymorphic IL-1β residues are indicated above the ovine sequence in Fig. 3.17.

TABLE 3.4

Relationship of Ovine IL-1α and IL-1β cDNA Sequences to Other Species

	Interleukin-1α			Interleukin-1β				
	Nucleotide	Amino Acid		Nucleotide			Amino Acid	
Species	Coding	Pro	Mat	5' UTR	Coding	3' UTR	Pro	Mat
Bovine	97	97	98	97	96	85	95	96
Porcine	88	83	80	na	na	na	na	na
Human	81	72	69	81	78	59	62	64
Rabbit	80	70	64	67	76	49	63	63
Rat	80	63	57	75	72	43	59	62
Murine	80	61	54	66	70	45	56	59

Table 3.4

Comparisons are listed as % identities with respect to the ovine sequences. Amino acid figures were obtained from comparison of the cDNA translation products. Sequences were optimally aligned using the method of Needleman and Wunsch, (1970). At the nucleic acid level, the ovine IL-1α untranslated sequences were not obtained. Only coding sequence comparisons are therefore given.

UTR - untranslated sequence; Pro - proprotein; Mat - mature protein; na - not available

FIGURES 3.16 AND 3.17

IL-1 Sequence Comparisons

Published IL-1 sequences are aligned relative to the respective ovine sequences.

Dots indicate conserved residues. Potential glycosylation sites are underlined. The position of the start of the mature proteins, Ser-120 in IL-1α and Ala-114 in IL-1β, is indicated by **V**. Potentially polymorphic IL-1β residues which were detected in this study are shown above the ovine sequence in Fig. 3.17.

References:

Bovine IL-1α and IL-1β:	Maliszewski et al., 1988; Leong et al., 1988
Porcine IL-1α:	Maliszewski et al., 1990
Rabbit IL-1α and IL-1β:	Furutani et al., 1985,1986
Human IL-1α and IL-1β:	March et al., 1985
Murine IL-1α and IL-1β:	Gray et al., 1986
Rat IL-1α and IL-1β:	Nishida et al., 1988a

FIGURE 3.16

IL-1α Sequence Comparisons

```
Ovine     1 MAKVPDLFEDLKNCYSENEDYSSEIDHLSLNQKSFYDASYEPLREDHMNK
Bovine      ..................................................Q...
Porcine     ..................E...D...................PG.G...
Rabbit      ............F....E...A...................H..C...
Human       ......M...........ED..S...........HV..G..H.GC..Q
Murine      ....................A................GS.H.TCT.Q
Rat         ..................E...A..............GS.H.NCTD.

Ovine    51 FMSLDTSETSKTSRLSFKENVVMVTA---NGKILKKRRLSLNQFITDDDL
Bovine      ............K.........A.---S...................
Porcine     ..P.S..K.......N..DS...AA.---...................
Rabbit      VV..S.....VSPN.T.Q....A...---S.................V..
Human       SV..SI.......K.T...SM.V.-.T--...V........S.S......
Murine      .V..R......M.NFT...SR.T.S.TSS...........FSETF.E...
Rat         .V..R......M.TFT...SR.V.S.TSNK..........F..PF.E....

                                    V
Ovine    97 EAIANDTEEEIIKPRSAHYSFQSNVKYNFMRVIHQEDILNDALNQSIIRD
Bovine      ....N.............................................
Porcine     ....................T......M........NHQC.....................
Rabbit      .TNVS.P..G......VP.T..R.MR.KYL.I.K..FT.....LV..
Human       ....S...........PF..L.........I.KY.F.....-
Murine      QS.TH.L..-T.Q....P.TY..DLR.KL.KLVR.KFVM..S...T.YQ.
Rat         ....H.L..-T.Q....PH...N.LR.KLI.IVKQ.FIM..S...N.YV.

Ovine   147 MSGPYLTAATLNNLEEAVKFDMVAYVSE-EDSQLPVTLRISKYQLFVSAQ
Bovine      ........T...................-.............T.......
Porcine     P..Q..M..V....D.......A..T.N-D...........ETR......
Rabbit      T.DQ..R..P.Q..GD......G.....-...I.........QTP......
Human       ANDQ.....A.H..D.......G..K.SKD.AKIT.I.....T..Y.T..
Murine      VDKH..STTW..D.QQE.....Y..S.GGD..KY....K..DS.......
Rat         .DRIH.K..S..D.QLE.....Y..S.GGD..KY....KV.NT.......

Ovine   197 NEDEPVLLKEMPETPKIIKD-ETNLLFFWEKHGSMDYFKSVAHPKLFIAT
Bovine      ....................-.............................
Porcine     ..........L.....T...-..S.........N......A.........
Rabbit      ..............R..T.S.SDI.....TQ.NKN....A...Q.....
Human       D..Q............T.TGS.........T..TKN..T.....N.....
Murine      G..Q......L.......TGS..D.I...KSIN.KN..T.A.Y.E.....
Rat         G..K......I.....L.TGS..D.I.....IN.KN..T.A.F.E.L...

Ovine   246 KQEKLVHMASGPPSITDFQILEK*
Bovine      ......................*
Porcine     R........P.L..V.......NQS*
Rabbit      .P.H.....N.L..M.....S*
Human       ..DYW.CL.G............NQA*
Murine      .EQSR..L.R.L..M.....S*
Rat         .EQSQ..L.R.L..MI....S*
```

FIGURE 3.17

IL-1β Sequence Comparisons

```
                          C
Ovine     1 MATVPEPINEVMAYYS-DENELLFEVDGPKQMKSCTQHLDLGSMGDGNIQ
Bovine      ..........M.....-........A.D.......I.............
Rabbit      ......LTS.M...H.GN..D.F..A...NY....F.D...CCPDE.-..
Human       ..E...LAS.......GN.DD.F..A.......CSF.D...-CPL..G..
Murine      ......LNC..PPFD.-...D.F......QKN.G.F.TF...CP-.KS..
Rat         ......LNC.IA.FD.-E..D.F..A.R.QKI.D.F.A....CP-.ES..

               K         A
Ovine    50 LQISHQLYNKSFRQVVSVIVAMEKLR--SRAYEHVFRDDDLRSILSFIFE
Bovine      ......F.....................--NS..A...H.............
Rabbit      .R..C.P.....L..V..L....QKAVPCPQA.Q..G..TFF.L...
Human       .R..DHH.S.G...AA..V...D...KMLVPCPQT.QEN..STFFP....
Murine      ....Q.H.....A..L...V...WQLPVSFPWT.Q.E.MKTFF.....
Rat         ....Q.HLD....KA..L...V...WQLPMSCPWS.Q.E.PSTFF.....

                              V
Ovine    98 EEPVIFETSSDELL--CDAAVQSVKCKLQDREQKSLVLDSPCVLKALHLLS
Bovine      ............F.--...P...I.............A.............
Rabbit      ....LCN.WD.YS.--ECD..R.LH.R...AQ......SGTYE......NA
Human       ...IF.D.WDNEAY-VH..P.R.LN.T.R.SQ.....MSG.YE......QG
Murine      ...ILCDSWD.DDN-V..VPIRQLNYR.R.EQ......SD.YE......NG
Rat         ....LCDSWD.DD.LV..VPIRQLH.R.R.EQ..C...SD..E......NG

Ovine   147 QEMSREVVFCMSFVQGEERDNKIPVALGIRDKNLYLSCVKKGDTPTLQLE
Bovine      ...N.........................K...................
Rabbit      ENLNQQ...S........SND.......L.G........M.D.K......
Human       .D.EQQ...S........SND.......LKE........L.D.K......
Murine      .NINQQ.I.S.K.....PSND.......LKG......S.M.DG.......
Rat         .NI.QQ...S.......TSND.......LKG........M.DG.......

Ovine   197 EVDPKVYPKRNMEKRFVFYKTEIKNTVEFESVLYPNWYISTSQIEEKPVF
Bovine      ..............................................R...
Rabbit      S...NR...KK.......N.I...DKL....AGF.........T.YM...
Human       S....N...KK.......N.I..N.KL....AQF.........A.NM...
Murine      S....Q...KK.......N.I.V.SK.....AEF.........A.NE...
Rat         S....Q...KK.......N.I.V.TK.....AQF.........A.HR...

Ovine   247 LGRFRGGQDITDFRMETLSP*
Bovine      ..H..............*
Rabbit      ..NNS....LI.SF..FV.S*
Human       ..GTK........T.QFV.S*
Murine      ..NNS-....I..T..SV.S*
Rat         ..N-SN.R..V..T..PV.S*
```

3.7 Northern Blot Analysis of IL-1 mRNA Production in LPS Stimulated Alveolar Macrophages

IL-1 mRNA production by ovine alveolar macrophages on stimulation with 10mg/ml LPS was analysed by northern blot using the cloned cDNAs as probes. Ovine IL-1β m-RNA seems to be produced earlier than IL-1α m-RNA as seen in Fig. 3.18, the beta form showing a maximum at around 4h post induction as opposed to 6h for the alpha. IL-1β m-RNA has all but disappeared by 24h and IL-1α message is still detectable at 19h. This is similar to the profile of m-RNA production of human (Bernaudin et al., 1988) and murine (Chensue et al., 1991) IL-1s. Ovine IL-1 m-RNA molecular weights are approximately 2.0Kb (alpha) and 1.6Kb (beta), smaller than their bovine and human counterparts at approx. 2.2Kb and 2.0Kb respectively (Maliszewski et al., 1988). Blots of alpha and beta m-RNAs both also show faint bands hybridising at higher molecular weight, which may be partially spliced or unspliced RNAs.

Fig. 3.19 shows a northern blot of polyA$^+$ RNA and in the total RNA from which it was derived (4h LPS stimulation) probed with **β1** cDNA. The polyA$^+$ RNA shows a band at roughly the same position as in total RNA ie. at ~1.5Kb. A parallel blot probed simultaneously for IL-1β and TNFα showed no detectable 2.0Kb TNFα mRNA. This is in agreement with other studies in our laboratory which show that TNFα message is detectable at a maximum level 1-2h post LPS induction and thereafter rapidly disappears (Green et al., 1991). The sequence of production of these cytokine mRNAs by LPS stimulated ovine alveolar macrophages appears to be TNFα, IL-1β, IL-1α.

FIGURE 3.18

Northern Blots of IL-1 mRNA Production from LPS Stimulated Alveolar Macrophages

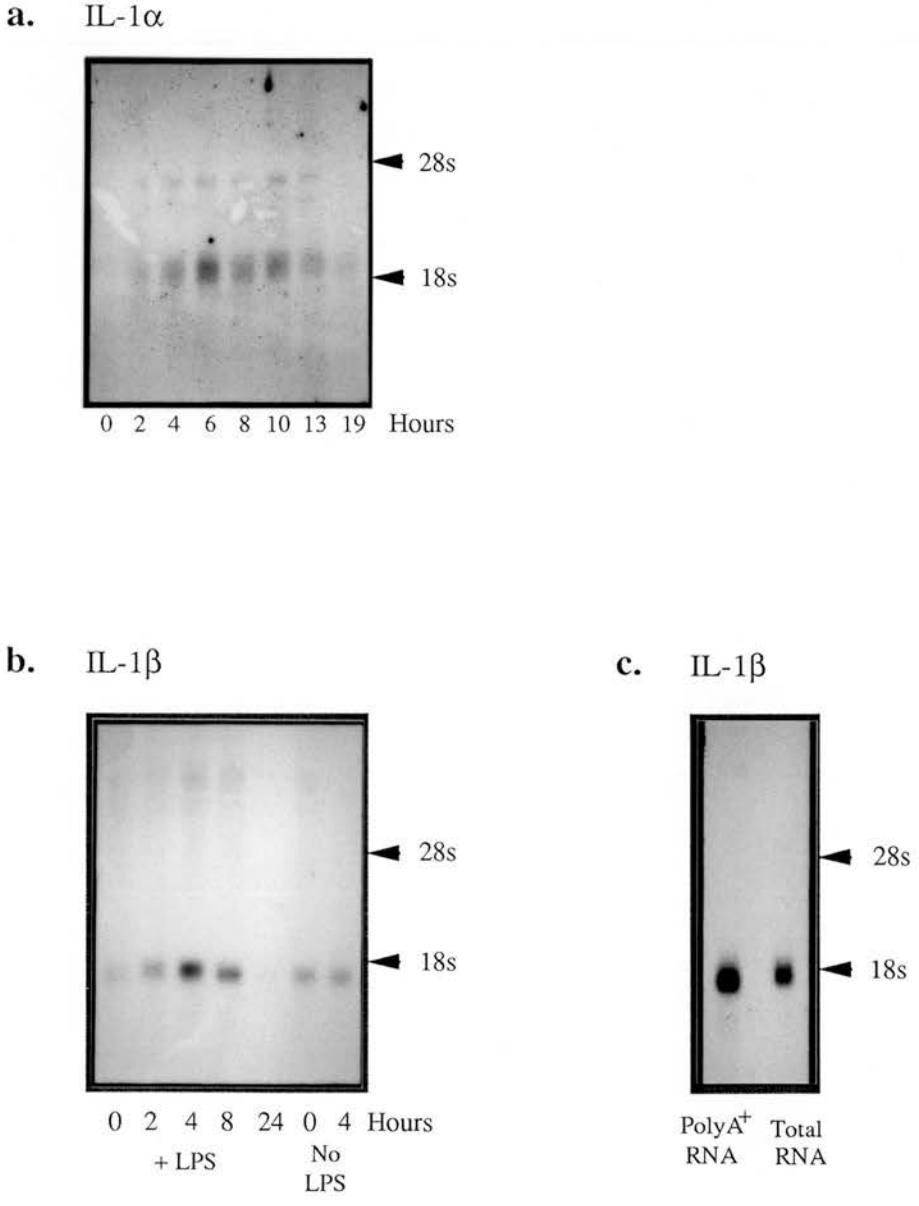


Figure 3.18.
a. and **b.** 10μg Total RNA extracted from alveolar macrophages after stimulation with 10μg/ml LPS for the times shown, was electrophoresed through denaturing agarose gels.
Northern blotted gels were hybridised with ^{32}P-labelled IL-1α (**a**) or IL-1β (**b**) cDNA probes.

c. 4μg polyA$^+$ RNA and 10μg total RNA from which the polyA$^+$ RNA had been selected, were electrophoresed as above and hybridised with ^{32}P-IL-1β cDNA

DISCUSSION

Although PCR has now become an extremely widely used and useful technique for isolating and amplifying DNA sequences, some of the problems which can be encountered, especially with respect to mispriming, became apparent during the amplification of ovine IL-1β. As a result, the cloning of ovine IL-1α by PCR was approached in such a way as to circumvent these problems.

The initial amplification attempted, of a central region within the IL-1β cDNA using mixed primers derived from consensus sequences in the human and bovine cDNAs, produced a product which appeared as a single strong band on electrophoresis. This band when cloned into pTZ18R revealed sequence with no clonal variations which was 96.8% and 78.2% homologous to the corresponding bovine and human sequences. Attempts to clone the outer ends of the molecule via anchored PCR were fraught with difficulty. Use was made of the IL-1β polyA tail in order to obtain the 3' sequence flanking the central clone and at the other end of the cDNA, the 5' leader sequence was extended by addition of an oligo-dA sequence using the terminal transferase reaction. Instead of the oligo-dT primer annealing to these polyA sequences in a specific 'anchored' fashion as anticipated, it appeared that the primer could also anneal to internal A-rich sequences.

The problems induced by this were two-fold. Firstly, PCR produced a large range of product sizes. It appears that concatamers can be formed, as these large products hybridise strongly with the **β1** central clone. Sequencing showed that both the 3' and 5' end cloned inserts obtained from such products were multimers. In addition, high molecular weight areas excised from smears on gels and used as templates for PCR with the intention of amplifying a large sized product, invariably produced a smear with a bias towards the lower molecular weight products. A concatamer containing primer binding sites along its length, would result in a range of product sizes, the smaller products effectively being preferentially amplified.

This phenomenon of oversize PCR products which produce smaller products on re-amplification, was also observed in our laboratory by Ian Green (personal communication) when using the technique of inverse PCR (Ochman et al., 1988). Inverse PCR involves circularising the cDNA and using outwardly pointing primers, complimentary to the ends of a known sequence within the cDNA. The resultant PCR, across the rest of the cDNA circle, should produce both 3' and 5' ends of the molecule in a single product. Green also obtained larger products than anticipated, which he postulated were the result of a "rolling

circle" type of mechanism operating during strand extension. The potential for concatamerisation, which may be greater when a monobasic primer is used, means that under these circumstances, products for cloning can not be selected on the basis of size.

Secondly, chimeric products could be formed as was detected during the attempted cloning of the 3' end of the cDNA, apparently as a direct result of using the homopolymeric oligo-dT as a primer. Clones obtained from the 3' region PCR smears yielded readable sequence followed by apparent compression over 78 bases then further sequence. This type of compression shows extension products present in all four lanes which did not mimic true compressions. Secondary structure was ruled out as being the cause of the compressions and on close inspection of the 3' sequence of the bovine IL-1β cDNA, it became apparent that there were a number of A-rich stretches which could potentially cause mispriming. As even the shorter oligo-dT was a 12-18bp mix (this being minimal for PCR primers), one can visualise the primer being able to anneal across these regions. One such section occurs at bases 482-506; AAGGAGAGGAAAGAGACAACAAGA which lies between and partially superimposed on the ovine nested primer sites B3.2 (bases 457-474) and B3.3 (bases 498-516). Oligo-dT mispriming in this region could potentially cause hindrance to the annealing of either or both B3.2 and B3.3 primers. Of probably greater significance, however, is the stretch of bases 715-727, which in the bovine sequence is AAATCGAAGAAAG. The ovine sequence was eventually found to contain A, not G, at base 727 which gives a 69% A content. (G=T base pairing is also stable which would increase this figure to 84% A + G content). The readable sequence from the 3' clones finished at base no. 721 which is in the middle of this proposed area of mispriming. Sequence 3' of this is only partially homologous with the subsequently determined ovine sequence and its origin is not obvious. This phenomenon of chimeric PCR products has been reported by other workers (Brakenhoff et al., 1991).

In retrospect, ligation of an oligonucleotide of known sequence to the cDNA termini, as suggested by Ohara et al, would be a more reliable basis for anchored PCR as the oligonucleotide could be tailored to ensure specific priming.

The final IL-1β cDNA sequence was obtained by choosing a bovine sequence primer from within the 3' untranslated region, which it was hoped would be reasonably specific. Although the untranslated sequences are not well conserved across other species, the bovine and ovine sequences are fortunately very similar (Table 3.4).

The original objective had been to obtain cDNA sequences by a PCR related cloning route, with the thought that library screening could be used to provide confirmatory sequence data. A λgt10 cDNA library was prepared and subsequent PCR reactions using positive λgt10 clones as templates and the B3.1/B5.3 primer pair, produced single bands on gels, which were the correct size and hybridised with **β3** (data not shown). These clones therefore probably contain complete 3' untranslated sequences and may contain full length cDNAs. These clones have not been pursued further.

PCR of IL-1α posed no problems even though the 3' primer site extended into the untranslated region. No problems were incurred as a result of having appended nucleotides at the 5' end of each primer which were required for cloning the product into the expression vector. The last four 'protective' bases were sufficient to allow efficient cutting by BamHI and subsequent direct ligation into BamHI cut pOGS40 vector. The PCR product cloned into pTZ18R/19R showed no sequence differences between any of the clones sequenced or the original PCR product.

The final accumulated ovine IL-1β cDNA sequence consisted of 32 bases of 5' leader, 801 coding bases and 145 bases of 3' untranslated sequence. The IL-1α cDNA consisted of 807 coding bases and 19 bases of the 3' untranslated sequence.

Comparison with other species, shows ovine and bovine IL-1 cDNA sequences to be very close, as expected. Porcine, human, rabbit, murine and rat IL-1s show decreasing degrees of homology with the ovine IL-1s. Homologies with respect to the three distinct cDNA regions, ie. 5' leader, coding and 3' untranslated sequences, are compared in Table 3.4 in the Results section. It can be seen that the 3' UTR is the most variable region although only 145 ovine nucleotides were available for inclusion in this analysis. Regions of translated sequences which are conserved across all other species are also generally conserved in the sheep although there are some notable differences. Chapter 6 will address conservation across specific regions within these sequences. The 25% identity between ovine IL-1α and IL-1β is similar to the human (26%), bovine (23%) and murine (22%) IL-1s (March et al., 1985; Maliszewsi et al., 1988; Gray et al., 1986).

Although ovine IL-1α and IL-1β sequences both display potential polymorphisms, IL-1β appears to be more variable than IL-1α. Three ovine IL-1β and two IL-1α ovine cDNA sequences have now been published. The IL-1α and IL-1β sequences published by Andrews et al. (1991) were derived by

screening a λgt10 cDNA library. The Seow et al. (1991) IL-1β coding sequence was PCR derived using one set of bovine sequence primers. Differences between these sequences and those presented here, give rise to one potential IL-1α and four potential IL-1β amino acid polymorphisms. It is of interest that all but one of the IL-1β differences are within the proprotein and not the mature protein coding sequences, which may be of significance considering that the IL-1β proprotein is supposedly not functionally active *in vivo*. The few sequence differences present are discussed below in relation to polymorphisms.

Potential ovine IL-1α polymorphisms

No differences within the IL-1α coding sequence were apparent from any of the clones sequenced here. There are, however, four nucleotide differences between this sequence and the ovine IL-1α sequence of Andrews et al. which represent potential polymorphisms. These differences are shown in Fig. 3.5 in comparison with the equivalent bovine bases, the two published bovine sequences being identical (Maliszewsky et al., 1988; Leong et al., 1988a). The resultant amino acid obtained on translation is given in brackets.

TABLE 3.5

Potential Ovine IL-1α Polymorphisms

Nucleo-tide	Amino acid	Ovine (Fiskerstrand)	Ovine (Andrews)	Bovine (Maliszewski/Leong)
153	51	T (Phe)	C (Phe)	T (Phe)
180	60	C (Ser)	T (Ser)	T (Ser)
220	74	G (Val)	A (Met)	G (Val)
285	95	T (Asp)	C (Asp)	T (Asp)

Table 3.5
Nucleotides and amino acids are numbered from the beginning of the ovine IL-1α pre-protein. Nucleotides in the Fiskerstrand sequences were PCR derived. Nucleotides in the Andrews' ovine sequence and the two bovine sequences were derived by λgt10 library screeing. Amino acids obtained on translation are given in brackets.

It can be seen that only one of these base changes gives rise to an amino acid alteration. Val-74 is conserved across all other species except porcine which has Ala-74. Fig. 3.5. The Andrews sequence was derived by library screening, which has a low rate of error incorporation. An ovine polymorphism is therefore likely at this point. Restriction digestion of genomic DNA with MaeIII and hybridisation with labelled IL-1α would confirm the presence of a G/A_{220} polymorphism. Given that none of the other base alterations result in amino acid changes, they may also represent polymorphisms. No enzymes are available for assessing these by digestion.

Polymorphisms have been reported for human IL-1α (Auron et al., 1984; March et al., 1986) giving rise to amino acid differences at positions 110 (Lys/Asn) and 114 (Ala/Ser), the latter of which is in the predicted mature protein. As both the human and the ovine amino acid substitutions are within regions showing high rates of evolutionary variation, the alterations probably carry no functional significance.

Potential ovine IL-1β polymorphisms

Polymorphisms seen in my PCR-derived IL-1β sequences are detailed in Table 3.1. Only three of the six nucleic acid differences give rise to amino acid differences. To assess the significance of these potential polymorphisms one must look at PCR error rates. The generally accepted average figure for PCR error is one change per 1500 bases, (similar rates have been measured in our own laboratory), which would mean an expected ten bases in a 15000 base pair sequence, ie. if all the nucleotide differences detected in IL-1β cDNA had been PCR induced, the rate of PCR error would be about that seen here. However, if some of the nucleotide differences detected in the IL-1β cDNA were due to PCR error rather than genuine polymorphism, one would expect a more random distribution, especially towards the 3' end of the coding sequence which was amplified via numerous independent but overlapping PCR reactions. No differences were however detected in this region although a few differences between the two published ovine IL-1β sequences were seen in the 3' untranslated region. From Table 3.1 it can be seen that each of the nucleotides detected, apart from C-74, was either found in clones derived from at least two independent PCR reactions, or appears to have a counterpart in the sequences from other species. Nucleotide C-74 is only present in clones derived from one particular PCR reaction. This nucleotide may therefore have been inserted as a result of PCR error. The other five alterations could represent genuine polymorphisms.

Even assuming a lesser error rate, one would also anticipate at least some PCR-induced error in the IL-1α cDNA clones. Not one difference was however detected.

Comparison the IL-1β sequences which I obtained with other published ovine sequences shows three amino acid differences with respect to the Andrews sequence and a further one with respect to the Seow sequence, as shown in Table 3.6. The Seow sequence also shows a TAA to TAG change. Apart from amino acid L/P145, the only differences between these sequences are at positions in which polymorphisms within my own clones were detected. The amino acid present in clone **β4**, which was used in expression studies, is indicated in bold print. Amino acids present at the same positions in other IL-1 sequences are appended at the bottom of the table for reference.

TABLE 3.6

Potential Amino Acid Polymorphisms Within Ovine IL-1β

Study	Nucleic acid / Amino acid				
Fiskerstrand	**A41/Y14**	**C163/Q55**	**T193/V64**	**T434/L145**	**A801/***
"	G41/C14	A163/K55	C193/A64		
Andrews et al.	A41/Y14	C163/Q55	T193/V64	T434/L145	A801/*
Seow et al.	A41/Y14	C163/Q55	T193/V64	C434/P145	G801/*
bo/h/rab	IL-1β = Y14	bo/mu/rat/rab	bo/rab = V64	bo = L145	
	mu/rat = F14	= Q55	h/mu/rat = A64	mu/rat/rab = N145	
all species	IL-1α = C14	h = H55		h = Q145	

Table 3.6
Sequences from all PCR derived ovine IL-1β clones obtained in the study being reported here, were compared with the two other published ovine IL-1β sequences. Differences are shown as the nucleotide and its position / amino acid obtained on translation. 'PCR polymorphisms' are shown separately under the Fiskerstrand study and the clone used for expression of the IL-1β protein is given in bold print. Amino acids present at these positions in other IL-1 sequences are indicated below the double line for reference. The stop codon is indicated by *.

One method of assessing potential polymorphisms is by sequence comparison across species. By this criterion, differences at three of these residues could represent true polymorphisms (see Table 3.1). There is no precedent for residue K55 but the nucleotides coding for this amino acid were present in clones derived from separate PCR reaction sets. PCR induced error therefore seems highly improbable. This amino acid change would also represent a moderately conservative substitution.

Restriction digests of genomic DNA could confirm the presence of polymorphisms by yielding different patterns of digestion products. Unfortunately, however, there are no restriction enzyme sites which encompass the suspected polymorphic sites in ovine IL-1β cDNA.

IL-1 mRNA production by macrophages

Northern blot analysis of the production of IL-1 by LPS stimulated ovine alveolar macrophages over 24h suggests that IL-1β mRNA is produced no later than IL-1α mRNA. IL-1β mRNA appears to peak at about 4h and IL-1α mRNA around 6h. The exact sequence of events can not be deduced as this data was derived from separate experiments. Andrews et al. have suggested that ovine IL-1 alpha is produced earlier than beta. The main differences between the two ovine studies are the length of time for which alveolar macrophages were allowed to adhere to culture dishes prior to LPS stimulation and the culture medium used, both of which could affect the mRNA levels detected. The LPS dosage, 10μg/ml, was the same in both studies. Nash stimulated the macrophages in RPMI medium containing 10% foetal calf serum ($RPMI_{10}$) after allowing the cells to adhere for 1 hour. In my experiments, the macrophages were allowed to adhere and stabilise in Iscoves serum free medium for 16h prior to stimulation, the reasons being that the process of macrophage adhesion is known to stimulate IL-1 mRNA synthesis even though translation is not induced. It is also known that adhesion and LPS can induce IL-1 mRNA simultaneously via independent mechanisms (Labadia et al., 1990), so that in effect, more IL-1 mRNA would be detected from cells not allowed to recover from the adhesion process before prior to LPS stimulation. In addition, heat inactivated serum has also been shown to induce greater amounts of IL-1α than IL-1β from human macrophages (Lonneman et al., 1989 and data not shown). In support of the effect of serum on IL-1 mRNA induction in sheep, I initially found that macrophages incubated overnight in $RPMI_{10}$ contained readily detectable amounts of IL-1 mRNA, prior to LPS stimulation. In contrast, after 16h incubation in Iscoves serum free medium, only traces of the mRNAs were detectable by northern blotting. It could be, therefore, that the effects of both adherence and foetal calf serum are leading to the different induction patterns

seen in the two laboratories. Northern blot data from unstimulated samples in the Nash study, in which both IL-1 mRNAs are seen to be present at 5h but have disappeared by 20h, may substantiate this idea.

In conclusion, the ovine IL-1 cDNAs have been cloned into both sequencing and expression vectors, and the methodology by which these sequences were obtained has been appraised. Potential nucleic acid polymorphisms have been detected in ovine sequences, 1 in the mature IL-1β, 6 in the IL-1β proprotein and 4 in the IL-1α proprotein. Of these, 1 in IL-1βm, 3 in IL-1βp and 1 in IL-1αp lead to amino acid differences. There is only 25% identity between ovine IL-1α and IL-1β, the same as is seen in other species. The coding regions of both sequences show high levels of homology with their bovine counterparts and on translation show retention of motifs conserved across all other species. Initial comments have been made on the significance of some regions within the sequence. The importance of conserved regions with respect to structure, receptor binding and biological activity, will be addressed in chapter 6.

CHAPTER 4

EXPRESSION AND CHARACTERISATION OF RECOMBINANT OVINE INTERLEUKIN-1α AND INTERLEUKIN-1β

INTRODUCTION

Human, murine and bovine IL-1s have all been expressed as recombinant proteins and both prokaryotic and eukaryotic expression systems have been successfully used for this purpose. *E. coli* has been the most frequently used host but rIL-1 has also been expressed in transfected monkey COS cells, murine L cells and yeast, as well as by *in vitro* translation in rabbit reticulocytes and Xenopus laevis oocytes (see Chapter 1, Sect 1.7.2). In some cases the rIL-1 has been synthesised fused to hybrid secretory sequences for the study of its secretion and activity (Rosenwasser et al., 1986; Casagli et al., 1989; Pecceu et al., 1991). The choice of expression vector and host cell depends on various factors including expected yields of protein, ease of purification and possible posttranslational modification of the protein.

At the time when ovine IL-1 was to be expressed as recombinant protein, the yeast Ty-vlp expression system was a comparatively new system, produced by British Biotechnology Ltd., which had only been used for the production of viral proteins. In this system, *Saccharomyces cerevisiae,* strain BJ2168, transformed with vectors based on the yeast retrotransposon Ty, yields fusion proteins which self-assemble into virus like particles (vlps) from which the recombinant protein can be enzymatically cleaved. Studies on recombinant HIV proteins and Interferon (Adams et al., 1987a; Malim et al., 1987; Kingsman et al., 1987; Griffiths et al., 1991) had shown the following attractive features of the Ty expression system;

(i) the ability to produce a range recombinant proteins from 3-42 kD
(ii) high yields of biologically active recombinant protein
(iii) ease of purification of the recombinant protein
(iv) increased immunogenicity of the recombinant protein when presented in the arrayed, multivalent form of the vlp
(v) recombinant proteins expressed in this system have been shown to be antigenically authentic.

FIGURE 4.6

Growth of Transformed *Saccharomyces cerevisiae* Strain BJ2168

a. pMA5620 transformant for constitutive p1 protein production

b. pOGS40/IL-1 transformant pre-induction

c. pOGS40/IL-1 transformant post galactose induction

Samples from growing cultures were viewed under a light microscope and photographs were taken at x50 magnification.

These features fulfilled the requirements for recombinant ovine IL-1 and in addition, the system was currently being successfully used in our own laboratory for production of maedi-visna virus p25 core protein (Reyburn et al., 1992).

A brief description of the essential components of this expression system follows. Yeast Ty is a member of a class of eukaryotic DNA retrotransposons. The haploid genome of laboratory strains of yeast contains 30-35 copies of Ty which are found at variable chromosome locations (Kingsman and Kingsman, 1988). The major Ty RNA, which is also the intermediate in Ty transposition, is 5.7kb in length and consists of two open reading frames, TYA and TYB. TYA is expressed by simple translation to produce p1, a 50kD protein (Mellor et al., 1985a; Adams et al., 1987b). TYB is expressed as a fusion with the TYA gene resulting in a 190kD TYA:TYB fusion protein, p3. TYA is further processed to smaller proteins, p2, p4, p5 and p6. The transposition cycle of Ty involves the packaging of Ty RNA into pre-Ty-vlps comprised of p1 and p3, followed by proteolytic maturation due in part to the action of the TYB-encoded protease. The vlps also contain reverse transcriptase, a tRNA primer and probably integrase which enable the vlps to carry out an endogenous reverse transcription to produce a double stranded copy which integrates into the genome (Mellor et al., 1985b). Although p2 is the major protein in mature vlps, the p1 to p2 proteolysis is not necessary for particle formation. It is therefore possible to construct hybrid Ty-vlps from p1 fusion proteins, which are polyvalent derivatives of the added protein. The specialised expression vectors pMA5620 and pOGS40, illustrated diagramatically in Fig. 4.1a,b, have been designed to include elements required for recombinant protein:p1 vlp expression (Adams et al., 1991).

Plasmid pMA5620 consists of an expression cassette inserted into an *E. coli* yeast shuttle vector. The expression cassette consists of the yeast phosphoglycerate kinase (PGK) promoter flanking the TYA gene, TYA(d), which has been truncated at codon 381 by addition of a BamHI site, for insertion of additional cDNA. Termination codons in all three reading frames and a transcription terminator follow the BamHI site. The shuttle vector contains origins of replication for growth in both *E. coli* and yeast, the gene for ampicillin resistance for selection in *E. coli*, and a LEU2 gene for selection in yeast strains which are auxotrophic for leucine production. The plasmid copy number is maintained at 100-200 copies per cell. In the current study, this vector was only used for constitutive production of p1 protein as a control preparation.

Plasmid pOGS40 is derived from pMA5620 and contains a hybrid PGK-GAL promoter (PAL). pOGS40 is used for production of the fusion protein by

insertion of the required cDNA sequence within the 3' end of the TYA(d) sequence. A Factor Xa (FXa) restriction protease recognition sequence is appended at the 5' terminus of the inserted cDNA. p1 or p1-fusion genes are only expressed from the PAL promoter following induction by galactose. The resultant fusion proteins self-assemble into vlps consisiting of a yeast p1 protein core fused to recombinant protein on the outside with the FXa recognition site as a bridge (Fig. 4.2). Inducible expression is advantageous because in general, induction by galactose results in greater yields of fusion proteins than obtained by constitutive production. In addition, allowing the yeast to grow to a high density prior to switching on fusion protein production is important if the recombinant protein is liable to be toxic to the cells.

The yeast host strain, *S. cerevisiae* BJ2168, is a protease deficient strain which minimises degradation of the vlps by intracellular proteases. The strain carries a mutation in the GAL2 gene, (a gene normally encoding a permease which allows galactose to enter the cell), but as some galactose can enter the cell via a constitutive pathway, the strain is useful when galactose induction of expression is required.

In the presence of galactose, GAL4 protein is released, binds to the upstream galactose activation sequence of the PAL promoter and activates transcription of the TYA gene. One of the main constraints of the galactose induction system is the low level of GAL4 gene expression especially if the cell contains multiple copies of the plasmid. The plasmid pUG41S contains the structural gene for GAL4 under the control of the GAL1 promoter and which is over expressed in the presence of galactose. Co-transformation of pUG41S as a helper plasmid can therefore act to increase fusion protein yields. pUG41S also contains a uracil selection module. The BJ2168 yeast strain can therefore be transformed to leucine independence with either pMA5620 or pOGS40 plasmids and to both leucine and uracil independence if both pOGS40 and pUG 41S plasmids are present.

The production and processing of IL-1 has been described in detail in Chapter 1 Sect 1.4. *In vivo,* the IL-1 proteins are produced as precursor molecules of M_r ~31kDa which are processed to yield the M_r ~17.5kDa mature proteins. IL-1α is active as both precursor and mature protein. Although IL-1β is generally considered to be biologically active only in its mature form, recombinant human proIL-1β, either expressed in COS monkey cells or synthesised by *in vitro* translation, has been reported to be biologically active in D10.G4.1 assays and on fresh thymocytes (Rosenwasser et al., 1986; Andrews et al, 1992; Jobling et al., 1988) although to a much lesser degree than the mature protein.

FIGURE 4.1

Vectors used for Transforming *S. cerevissiae* Strain BJ2168 in the Yeast Ty-vlp Expression System

A. pMA5620 expresses yeast p1 protein constitutively from the TYA(d) gene under the control of the phosphoglycerate kinase (PGK) promoter.

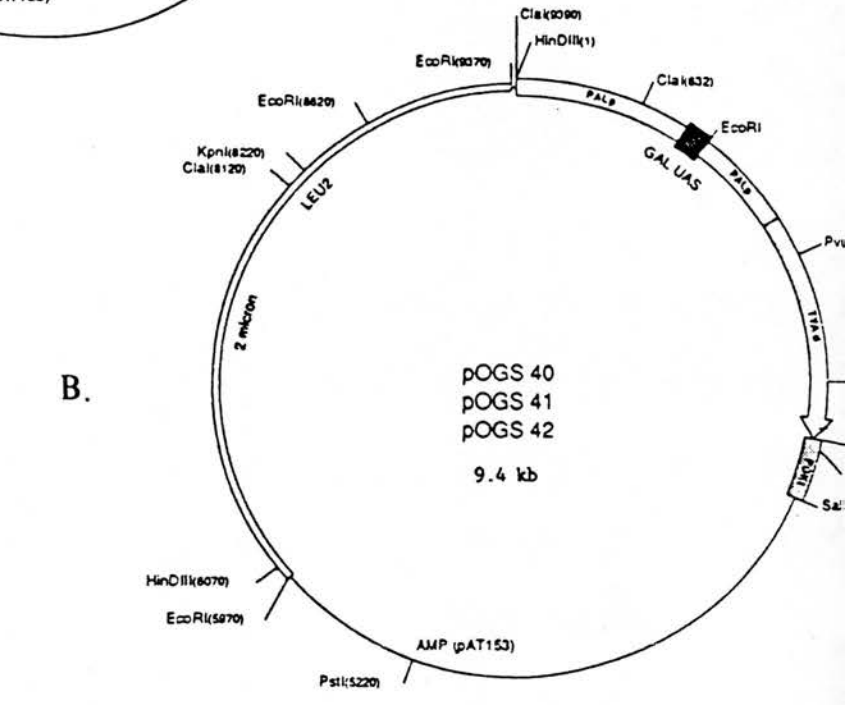
B. pOGS40 is a derivative of pMA5620 which requires galactose induction for protein expression. cDNA sequences are inserted into the vector via the BamHI restriction enzyme site within the TYA(d) gene. Fusion proteins are expressed under the control of a hybrid PGK/GAL (PAL) promoter.

pMA5620 and pOGS40 plasmids are *E. coli* / yeast shuttle vectors each of which contains an Ampicillin resistance gene (AMP) for selection in *E. coli* and a Leucine selection module (LEU) for growth in yeasts auxotrophic for Leu biosynthesis.

C. pUG41S expresses the GAL4 gene in the presence of galactose and under control of the GAL1 promoter. The plasmid contains a Uracil selection module.

Restriction enzyme sites are indicated on all vectors. Details with respect to vector usage are given in the main text.


A.
pMA 5620
pMA 5621
pMA 5622
9.4 kb
ClaI(9090)
HinDIII(1)
EcoRI(9370)
ClaI(632)
EcoRI(8420)
KpnI(8220)
ClaI(8120)
LEU2
2 micron
PVU II(1700)
TRP1
PstI(2250)
BamHI(2650)
ClaI(2700)
SalI(2950)
HinDIII(6070)
EcoRI(5970)
AMP (pAT153)
PstI(5220)


B.
pOGS 40
pOGS 41
pOGS 42
9.4 kb
ClaI(9090)
HinDIII(1)
EcoRI(9370)
ClaI(632)
PAL9
EcoRI
GAL UAS
EcoRI(8420)
KpnI(8220)
ClaI(8120)
LEU2
2 micron
TRP1
HinDIII(6070)
EcoRI(5970)
AMP (pAT153)
PstI(5220)

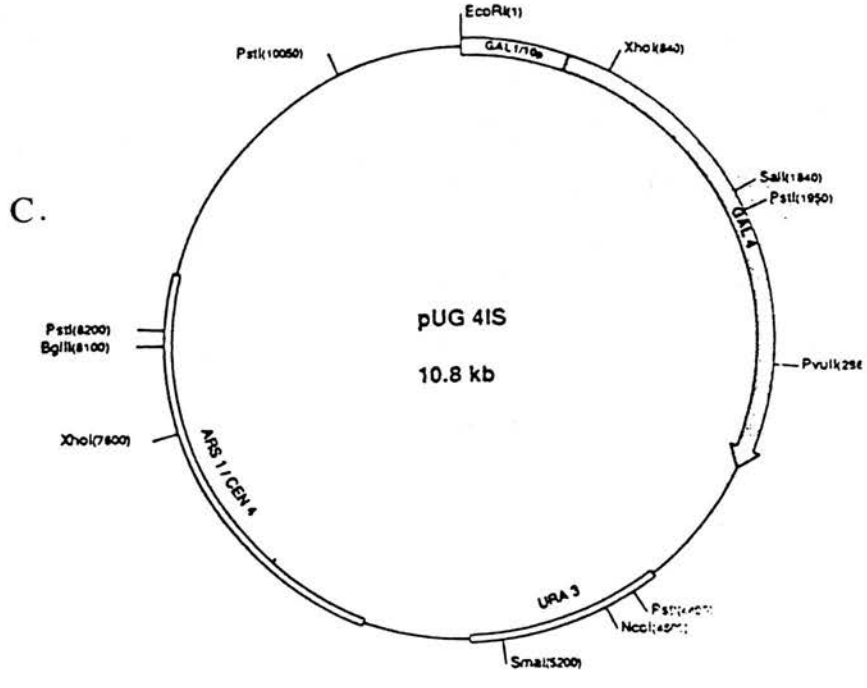
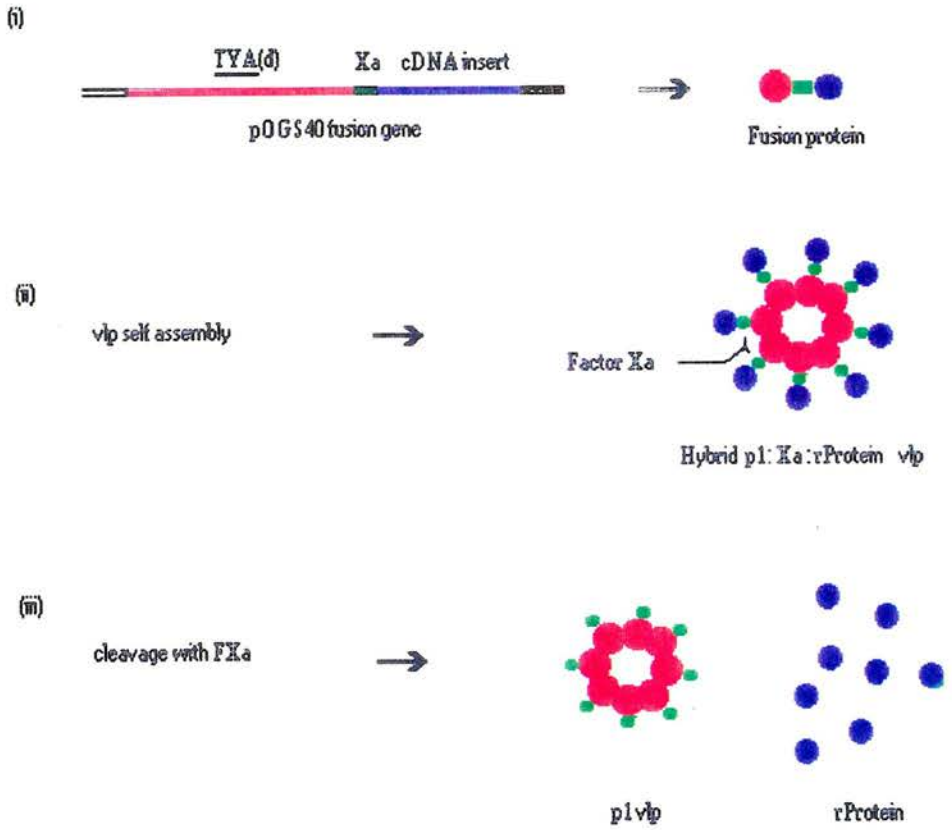

C.
pUG 41S
10.8 kb
EcoRI(1)
PstI(10050)
GAL1/10p
XhoI(840)
SalI(1840)
PstI(1950)
GAL4
PvuII(25
PstI(8200)
BglII(8100)
XhoI(7600)
ARS1/CEN4
URA3
SmaI(5200)

FIGURE 4.2

Production of Recombinant Proteins by the Yeast TY-vlp Expression Syste



(i) Galactose inducible fusion protein is expressed from pOGS40 constructs under the control of the hybrid PGK/GAL (PAL) promoter. Each construct contains, 5' to 3', the TYA(d) gene for expression of yeast p1 protein (shown in red), a Factor Xa restriction protease coding sequence (shown in green) and cDNA sequence coding for the recombinant protein (shown in blue). The primary expression product is a fusion protein p1 : FXa : rProtein. (ii) Fusion proteins assemble into packed spherical virus-like particles (vlps) which when cleaved with Factor Xa, (iii), release the recombinant protein while retaining their p1 vlp structure.

For the purpose of determining the biological activity of ovine IL-1, pOGS40 expression constructs for precursor and mature forms of both IL-1α and IL-1β have been produced as detailed in chapter 3. Briefly, PCR derived cDNA was inserted into the vector via linkers containing BamHI restriction enzyme sites. The 5' end linkers included a sequence encoding the Factor Xa recognition site. CsCl purified DNA was prepared from 500ml cultures of selected *E. coli* JM83 colonies transformed with these constructs. Sequencing of the double stranded pOGS40/IL-1-cDNA constructs confirmed that each of the inserts was in frame for correct protein synthesis and that the BamHI insert site had not been corrupted on ligation. Constructs were transfected into *S. cerevisiae* strain BJ2168 either with or without pUG41S as co-transformant.

A number of assay systems are available for determining IL-1 bioactivity. Of these, the two most specific and frequently used assays employ the murine cell lines EL4.NOB1 and D10.G4.1 or derivatives thereof. In view of suspected species barriers there was a possibility that these cell lines would not be of use in determining ovine IL-1 activity. Bioassays for IL-1 which are less specific because of being responsive to more than one cytokine, can however be effectively used for determining recombinant IL-1 activity ie. where there is no interference from cytokines such as TNFα. These assays involve either measuring thymocyte proliferation stimulated by IL-1 with Phytohemagglutinin (lectin extracted from *Phaseolus vulgaris)* as co-mitogen or measuring glycosylaminoglycans released from cartilage as a result of the degradative action of IL-1. The basis of these assays has been detailed under 'actions of IL-1' in Chapter 1, Sect. 1.5. Because fresh tissue has to be employed for each assay, the reproducibility may not be as great as that obtained with cell lines, but the advantage of these assays is that homologous tissue can be used thus obviating any problems with respect to species specificity.

This chapter describes the successful expression of biologically active recombinant ovine IL-1α and IL-1β in the yeast Ty-vlp system. *S. cerevisiae,* strain BJ2168, transformed with pOGS40 / IL-1 vector constructs yielded rIL-1 expressed as yeast p1:rIL-1 fusion proteins from which the active rIL-1 could be cleaved and purified.

RESULTS

The expression of IL-1α and IL-1β as fusion proteins are detailed separately, Sections **4.1** and **4.2** respectively. Subsequent data is combined.

4.1 Expression of ovine IL-1β : yeast p1 fusion proteins

4.1.1 Transformation of *S. cerevisiae* BJ2168 with pOGS40/TYβ constructs

Five IL-1β preprotein (TYβp) and two mature protein (TYβm) constructs were transfected, into yeast. Transfectants and control transfectants were plated on Sc-glc (leu$^-$) plates in regeneration agar with additions as shown in Table 4.1.

Table 4.1

Growth of *Saccharomyces cerevisiae* Strain BJ2168 Transformed with IL-1β Expression Vectors

Vector	Insert	µg Construct	µg pUG41S	Additions	Transformant
pOGS40	TYβm1,2	7	+/-7	T, -/+U	1Bm - 6Bm
pOGS40	TYβp1-5	7-14	7	T, U	1Bp
pOGS40	–	4	+/-7	–	none
pMA5620	constitutive p1	4	–	T, U	pMA.p1
–	–	–	–	T, U, L	+
–	–	–	–	T	none
–	–	–	–	–	none

Table 4.1
BJ2168 yeast was transformed various constructs: pOGS40/IL-1β ± pUG41S for galactose induced expression of fusion proteins or pMA5620 for constitutive production of p1 protein for use as a control preparation. Amino acids were added as appropriate. Uracil and tryptophan were added for growth of single transformants. Double transformants only required added tryptophan. Untransformed cells only grew in the presence of added tryptophan, uracil and leucine.

U - Uracil; T - Tryptophan; L - Leucine; all at 2µg/ml
TYβm - cDNA coding for IL-1β mature protein, 2 different preparations transfected

Transformation efficiencies of IL-1β constructs were all very low. Six pOGS40/TYβm transformants (named 1Bm -> 6Bm) were obtained, all of which were much slower growing than pMA.p1. The Bm colonies were re-plated and incubated a further four days at 30°C then innoculated into Sc glc + T for small scale test culture. Phase contrast microscopy of transfected cultures showed the cells to be healthy and budding well at this stage. They are greenish grey, of diverse sizes and some contain large vacuoles. (see Fig. 4.6). Very few dead cells are seen. After subculture in galactose/minimal glucose medium, Sc-gal, to induce recombinant protein synthesis, the yeast cells were generally larger with a slight increase in the number of dead cells. Increased induction times resulted in marginally lower rather than higher yields of cells, which may be indicative of a slight toxicity of the vlps.

Only one IL-1β proprotein transformant, a double transformant called 1Bp, was obtained from five attempts at inserting the construct into yeast using three separate preparations of pOGS40/TYβp. 1Bp was initially even more slow growing than Bm transformants but the rate of growth increased after 4-5 days. Small scale liquid cultures were shaken in Sc-glc for seven days before the optical density was high enough to ensure sufficient growth under galactose induction conditions. By light microscopy the yeast cells looked healthy and were budding well. Most of the pOGS40/TYβp transformations were performed at the same time as successful transformations using other constructs. All pOGS40/TYβp construct preparations were CsCl purified and undegraded and the reasons for lack of transformant colonies are not obvious, although extremely slow growth may be a contributing factor. Under the microscope numerous small clusters of dividing cells were seen within the regeneration agar, but these did not develop into colonies of any useful size.

4.1.2 Selection of pOGS40/TYβ transformants for large scale culture

Crude protein preparations from non-induced and induced Bm and Bp transformants, pMA.p1 and untransformed BJ2168 were run on 10% reducing PAGE gels. Representative coomassie blue stained gels, show the appearance of a protein band at about 80kDa in the pOGS40/TYβp sample (Fig 4.3 a) and 68kDa in pOGS40/TYβm transformed samples. These bands are consistent with predicted sizes for fusion proteins consisting of p1 (M_r = 50kDa) plus IL-1βm (M_r = 17.5kDa) or IL-1βp (M_r = 31kDa) respectively. pMA5620 produces the expected 50kDa p1 band and no vlp bands are seen in the untransformed sample.

Electroblotted gels incubated with an anti-p1 polyclonal antiserum (Fig 4.3 b), show the presence of p1 or p1 fusion proteins in pMA5620 and pOGS40 vlp preparations respectively. Double transformants produced higher yields of βm:p1 vlps and 1Bm, was selected for large scale culture.

4.1.3 Preparative culture of IL-1β transformants and purification of vlps

Large scale cultures of 1Bm reached $OD_{600m\mu}$ of about 1.3 after 20h pre-induction and $OD_{600m\mu}$ =1.2 after 48h induction in 4l Sc-gal. 1Bp only reached an OD of 0.88 even after 66h induction. Light microscope pictures of IL-1β transformants pre- and post induction of BJ2168 are shown in Fig 4.6 combined with pictures of IL-1α transformants on pg *. Transmission electron microscopy reveals cells of diverse morphology and size. Induced cells contain variable numbers of vlps. Examples of 1B and 1A transformants are shown in micrographs Fig. 4.7. Data on growth of IL-1 and p1 transformants and resultant vlp yields are combined in Table 4.2 on pg 134.

Centrifugation of crude cracked yeast supernatant onto a 60% sucrose cushion yielded a thick, particulate, ochre coloured interface consisting mainly of vlps but also some yeast protein. Samples from the supernatant, interface and cushion fractions were run on SDS-PAGE gels and showed that the vlps are in fact distributed throughout. Fig 4.4 (lanes 1-4) compares the relative amounts of vlps and yeast proteins in untransformed samples, crude extracts of transformed samples and the interface and cushion fractions of partially purified preparations. Up to 4ml of pooled interface and cushion layers was centrifuged through 34ml 15 - 45% linear sucrose gradients over a 60% sucrose cushion at 50,000g for 3 hours at 4°C and sequential 2ml fractions collected from the top of the gradient. Coomassie stained 10% SDS-PAGE gels show the distribution of βm:p1 and βp:p1 vlps within these gradients (Fig 4.4 lanes 6-19). The bulk of the contaminating yeast proteins remain in the upper fractions of the gradient, with the remainder being distributed fairly evenly throughout the vlp containing fractions. Sedimentation velocities of βm:p1 vlps were 30s to 300s, and of the larger βp:p1 vlps were 200s to 500s. Transmission electron microscopy revealed a large range in size of the vlps, many of which are very irregularly shaped. Fig. 4.7 shows examples of βm vlps both within transformed yeast cells and after purification. P1 vlps in contrast are regular and are seen at a greater density within yeast cells (the latter is not shown).

FIGURE 4.3

Fusion Protein Expression from BJ2168 Transformed with TYβ Constructs

(a) Coomassie stained 10% SDS-PAGE gel of crude extracts from BJ2168 yeast preparations showing the appearance of p1 at 50kDa, βp:p1 fusion proteins at 81kDa and βm:p1 fusion proteins at 68kDa.
Lane 1, untransformed; Lane 2, transformed with PMA5620; Lane 3, transformed with pOGS40/TYβm; Lane 4, transformed with pOGS40/TYβp.

(b) Electroblotted 10% SDS-PAGE gel reacted with anti-p1 polyclonal antiserum showing the presence of p1 in all transformed samples.
Lane 1, untransformed; Lane 2, p1 vlp; Lane 3, βm:p1 vlp; Lane 4, βp:p1 vlp.

FIGURE 4.4

Purification of IL-1βm IL-1βp Fusion Proteins

(a) βm:p1; (b) βp:p1.
Coomassie stained 10% SDS-PAGE gels of crude extracts from pOGS/TYβm and pOGS/TY/βp transformed BJ2168 and sucrose gradient purification profiles of the vlps. Lane numbers apply to both fusion proteins.

Lane 1, untransformed BJ2168; Lane 2, transformed BJ2168; Lane 3, interface vlps; Lane 4, cushion vlps. Lane 5, molecular weight markers; Lanes 6-19, vlps in successive fractions after sucrose gradient purification of pooled interface and cushion samples. Gels of 20μl aliquots from βm:p1 gradient fractions and 5μl aliquots from βp:p1 fractions are shown. Sucrose concentration across the gradient is indicated.

Two aspects of vlp recovery had to be taken into consideration, purity and concentration. Processing the the interface and cushion vlps separately did not result in a greater degree of vlp purity. Neither did longer or faster centriguagtion during the cushioning step which rather resulted in pelleting of the vlps with subsequent reduction in recombinant protein yields. Gradient purified samples can be diluted up to fifteen-fold which is not ideal for preparation already at a relatively low concentration. In an attempt to increase both concentration and purity of the vlps, various 15ml 15-60% discontinuous sucrose step gradients were run in place of the 43ml linear gradient. These gradients did not however appear to confer substantial benefit and were not routinely used in vlp purifications. The purity problem was judged to be the result of the comparatively low yield of βm:p1 vlps. Gradient fractions containing the highest concentration of vlps were therefore retained as optimal, and individual fractions were pooled or not, as required.

4.2 Expression of ovine IL-1α : yeast p1 Fusion proteins

4.2.1 Transformation of *S. cerevisiae* BJ2168 with pOGS40/TYα Constructs

Constructs for both forms of the IL-1α protein were transfected into yeast BJ2168 either with or without pUG 41S as co-transfectant. A number of transformants were obtained for each IL-1α construct, all of which grew well, as opposed to those containing IL-1β constructs. A coomassie stained 10% PAGE gel of crude extracts (Fig 4.5 a) shows that IL-1αp vlps (αp:p1) appear to be produced in greater amounts than IL-1αm vlps (αm:p1). Co-transfection with pUG 41S enhanced production of αm:p1 slightly, as is also shown by a western blot against polyclonal anti-p1 antiserum (Fig 4.5 b).

4.2.2 Preparative Culture of IL-1α Transformants and Purification of vlps

Selected colonies, 1Ap and 1Am, were grown in large scale culture. 1Am growth was comparable to 1Bm and 1Ap was much faster growing than 1Bp. By light microscopy there are no obvious differences between pOGS40/TYα and pOGS40/TYβ transformed yeasts either before or after induction. Fig. 4.6 shows the relative sizes of uninduced transformants (b) and transformants under conditions of vlp production (a, c). Electron micrographs of 1A, 1B and pMA.p1 transformants are similar, both showing great morphological diversity (Fig. 4.7).

IL-1α vlps were cushioned onto 60% sucrose but because yields of both IL-1αp and IL-1αm vlps were greater than IL-1βm vlps, it was found to be not necessary to run the cushioned extracts on sucrose gradients in order to obtain vlp preparations of equal purity (85-95%). The advantage of this was that the vlps were retained at higher concentrations. The concentration of cushion vlps is typically 10 times that of sucrose gradient purified material.

FIGURE 4.5

Fusion Protein Expression from BJ2168 Transformed with TYα Constructs

a.

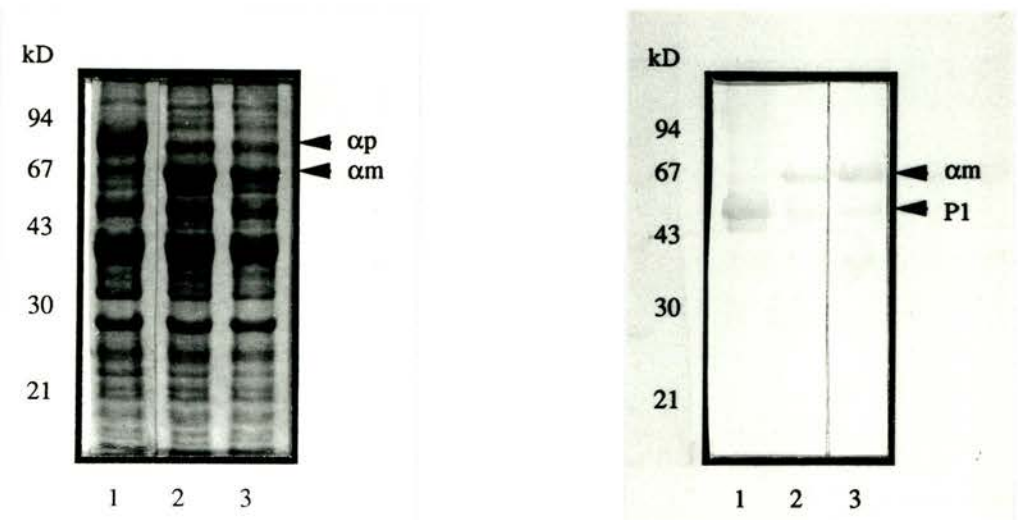


Figure 4.5

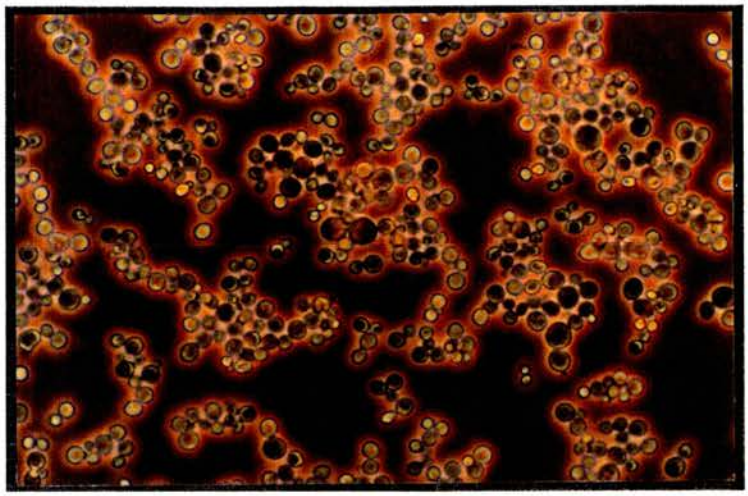
a. Coomassie stained 10% SDS-PAGE gel of 5µl aliquots of crude extracts from single or double transformants, showing the appearance of αp:p1 fusion proteins at ~81kDa and αm:p1 fusion proteins at ~68kDa. By way of comparison, p1 runs at 50kDa on 10% SDS-PAGE gels.

Lane 1, αp:p1 vlp, pOGS40 single transformant; Lane 2, αm:p1 vlp, pOGS40/pUG41S double transformant ; Lane 3, αm:p1 vlp, pOGS40 single transformant.

b. The presence of p1 in αm:p1 vlps blotted against polyclonal anti-p1 antiserum at 1/2000 dilution shows that co-transformation with pUG41S increases αm:p1 yields slightly.

Lane 1, p1 pMA5620 transformant; Lane 2, αm:p1 single transformant; Lane 3, αm:p1 double transformant.

a.



b.

c.

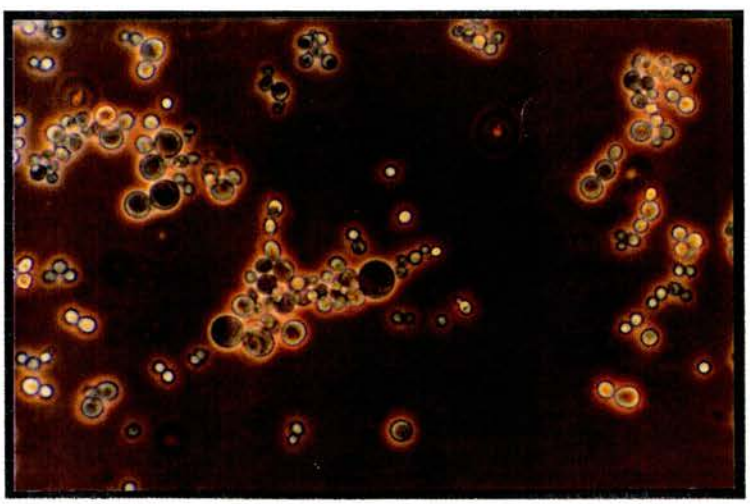
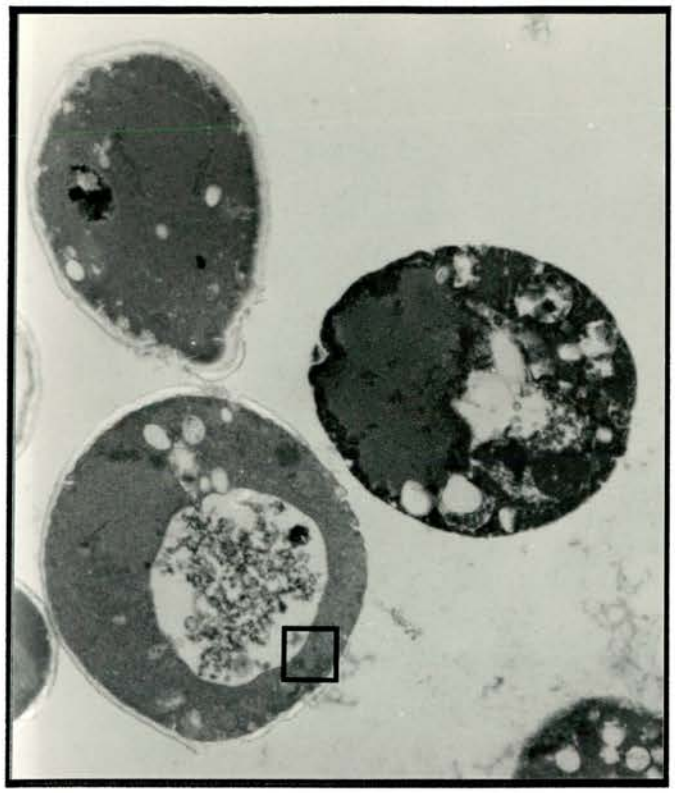
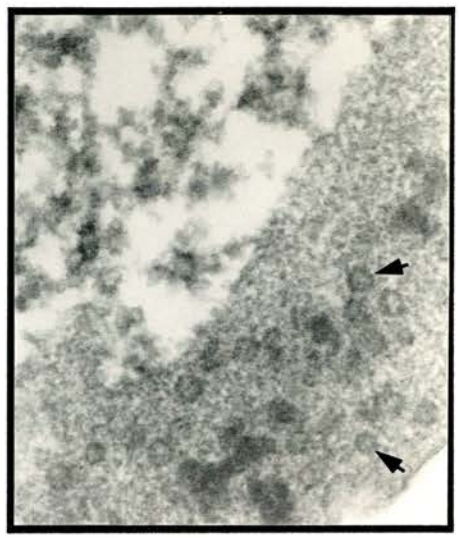


FIGURE 4.7

Electron Microscopy of Yeast Transformants and Purified Fusion Proteins (Vlps)

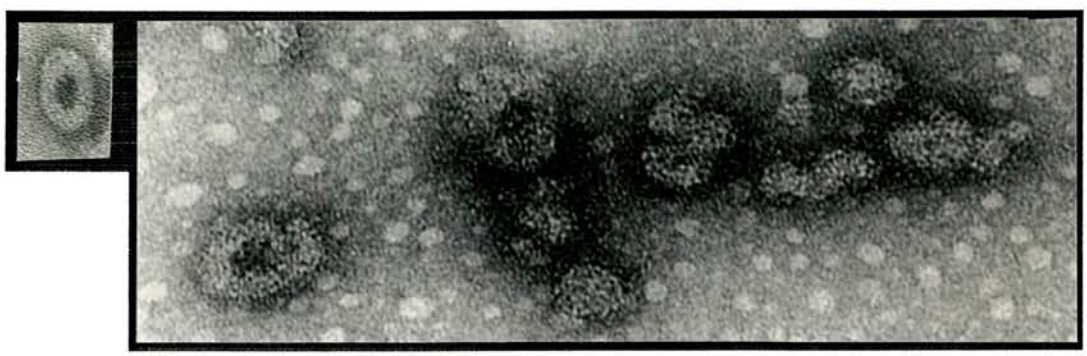


Yeast (x 7200)



Vlps within yeast cell (x 71000)

p1 vlp
(x 154000)



Purified βm:p1 vlps (x 330000)

Figure 4.7
pOGS40/IL-1βm yeast cells under conditions of vlp production and preparations of purified IL-1βm:p1 fusion proteins were treated as described in Chapter 2.10 and viewed by Transmission Electron Microscopy. A p1 vlp is shown as an inset for comparison with the βm:p1 vlps. Magnifications are shown on the individual components of the composite figure.

4.3 Comparison of IL-1α and IL-1β Fusion Protein Expression

The essential details of the production of the IL-1α and IL-1β fusion proteins are compared in Table 4.2. Prior to induction, all pOGS40/IL-1 transformants grew equally well, although more slowly than pMA.p1. Galactose induction reduced the growth rates, especially those of the proprotein transformants. This is in agreement with earlier observations on transformant growth rates on solid medium. Vlp production was independent of cell growth, ie. vlp production by 1Am, 1Ap and 1Bp were all comparable, ~0.2pg/cell, about half the amount of constitutive p1 vlp production, ~0.4pg/cell. In contrast, 1Bm vlp production, ~90fg/cell, was less than half that of the other IL-1 transformants despite good growth of the yeast, indicating a possible βm:p1 stability problem within the yeast. The amount of IL-1 within each of these vlp preparations is indicated in the column headed "Crude [IL-1]" where [IL-1] designates IL-1 concentration. Although vlp losses occurred at all stages of purification, the greatest αm:p1 and βm:p1 losses were during the 60% sucrose cushioning step, >40% of the vlps remaining in the 60% sucrose supernatant. <15% of the more dense αp:p1 and βp:p1 vlps were retained in this supernatant. Dialysis accounted for a ~10% reduction in yield.

TABLE 4.2

Growth of Transformed BL2168 in Large Scale Preparative Liquid Culture.
Fusion Protein Yields and Recovery of Recombinant Protein

Construct	Trans-formant	$OD_{600m\mu}$ Glc	$OD_{600m\mu}$ Gal	cells/ml Gal	Hrs. Gal	vlp yield mg/l	Crude$^{\Psi}$ [IL-1] mg/l	Total IL-1 recovered µg/l	Total IL-1 recovered %
pOGS/TYαp	1Ap	1.30	0.80	$2.7x10^7$	48	5.3	2.0	600#	53#
pOGS/TYαm	1Am	1.30	1.20	$4.0x10^7$	48	8.9	2.5	1000	45
pOGS/TYβp	1Bp	1.30	0.88	$2.9x10^7$	66	6.2	2.4	800	33
pOGS/TYβm	1Bm	1.20	1.20	$4.0x10^7$	48	3.7	1.0	300	30
pMA5620	*pMA.p1	1.50	na	na	na	20.0	na	na	na

Table 4.2
$OD_{600m\mu}$ = 1.5 represents $5x10^7$cells/ml; Cells were cultured in Sc-glc for 48 hours. Growth in Sc-glc inducing medium was as indicated. 20% glycerol stocks of all preparations were made prior to induction. [IL-1] represents the IL-1 concentration within unpurified vlps. Total IL-1 recovered represents the amount of IL-1 recovered after removal of cleaved vlps.
$^{\Psi}$ - theoretical yield; # – see below with respect to FXa cleavage, yield calculated on the basis of the size of the cleaved product actually recovered; * – constitutive production; na – not applicable.

4.4 Factor Xa (FXa) Cleavage of rIL-1 from Fusion Proteins

Vlps preparations dialysed into FXa buffer were cleaved for 24 hours at 22°C. From test cuts, a ratio of 1:100 FXa:vlp (w:w) and CHAPS and DOC at 0.05% (w/v) were established as the lowest concentrations required for efficient cleavage of all vlps. A western blot probed with anti-p1 polyclonal antiserum, shows the presence of p1 in the fusion protein, cleaved fusion protein and p1 only preparations, Fig. 4.8. p1 in the cleaved sample is slightly truncated with respect to native p1 because of the IL-1 coding sequence being inserted 5' of the p1 stop codon within the pOGS40/IL-1 vector construct.

FIGURE 4.8

Demonstration of the Presence of p1 Protein in Vlps

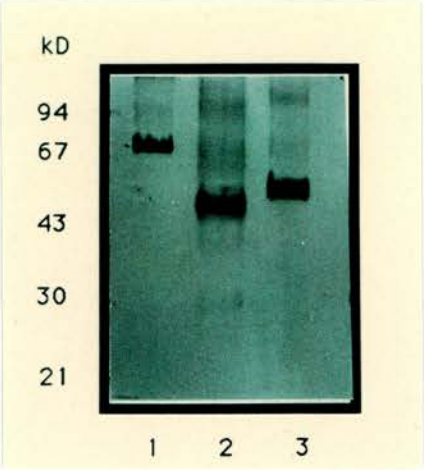


Figure 4.8
Western blot of fusion protein, cleaved fusion protein and native p1 vlps probed with anti-p1 antiserum at a dilution of 1/1000.

Lane 1, fusion protein; Lane 2, FXa digested fusion protein; Lane 3, p1.

FXa cleavage of αp:p1 and βm:p1 fusion proteins gave rise to unexpected products. The predicted molecular weight of the IL-1α proprotein, αp, is 31kDa. However, digestion of αp:p1 gave a main band running at about 21kDa on SDS-PAGE gels, just above that for the mature IL-1α protein. On digesting the βm:p1 vlp, two bands were consistently seen, but in varying proportions, running at about 20kDa and 18kDa, instead of the expected one band at less than 20kDa. (These cleavage products can be seen in Fig. 4.9). On inspection of the amino acid sequences it appeared that cleavage by the enzyme might have been occurring at "relaxed" sites. Table 4.3 shows motifs which have been found to be FXa cleavage recognition sites, the essential amino acid features being: *p1* basic, *p2* variable, *p3* charged and *p4* Ileu.

TABLE 4.3

Recognition Sites for Proteolytic Cleavage by Factor Xa

Substrate	Peptide recognition sequence (*p4 p3 p2 p1*)	Refererence
Prothrombin (bo)	IEGR-	Magnusson et al., 1975
Prothrombin (h)	IDGR-	Butkowski et al., 1977
β-globin	IEGR-	Nagai and Thorgersen, 1984
HIV p24 vlp	ISPR-	Gilmour et al., 1989
ovine IL-1α	IKPR- (position 110-113)	putative - current study
ovine IL-1β	IEEK- (position 240-243)	putative - current study

To test the possibility that the fusion proteins were being cleaved at internal IL-1 sites, the appearance of cleaved IL-1 was followed over 24 hours. Aliquots were removed from the digestions at time intervals and the reaction stopped by adding SDS/PAGE load buffer and boiling for 5min. The results, as shown by SDS-PAGE gels (Fig. 4.9), are consistent with the enzyme cutting at the supposed relaxed sites. Digestion of αp:p1 initially yields the expected 31kDa band with subsequent appearance of the 20kDa band and eventual loss of the larger band. βm:p1 digestion shows the initial appearance of the 20kDa band followed by the 18kDa band. On longer incubation the 20kDa band eventually disappears. The appearance of these bands was not affected by altering concentrations of CHAPS or DOC.

The apparent molecular weights of the recombinant ovine IL-1 proteins were larger than expected from the calculated sizes, i.e. ~20kDa as opposed to 17.5kDa for the mature proteins, in good agreement with data on IL-1 from other species which exhibit the same phenomenon (Maliszewski et. al., 1988). N-glycosidase treatment confirmed that this effect was not due to glycosylation of the recombinant proteins.

p1 vlps were removed from the digested samples by centrifugation at 100,000g for 90 minutes which unfortunately also removes a quantity of recombinant protein, possibly by adherence to the sticky vlps. The recombinant proteins at this stage were judged to be 80-95% pure and were used for activity studies and antiserum production. Examples from IL-1α preparations are shown in Figure 4.10. Recombinant IL-1 products, after spinning out the p1 vlp, are shown in Figure 4.11.

Final yields of rIL-1 at this stage are detailed in Table 4.2. For receptor binding studies the αm and βm preparations were further purified by HPLC (see below) which resulted in further losses of recombinant protein at each dialysis step.

FIGURE 4.9

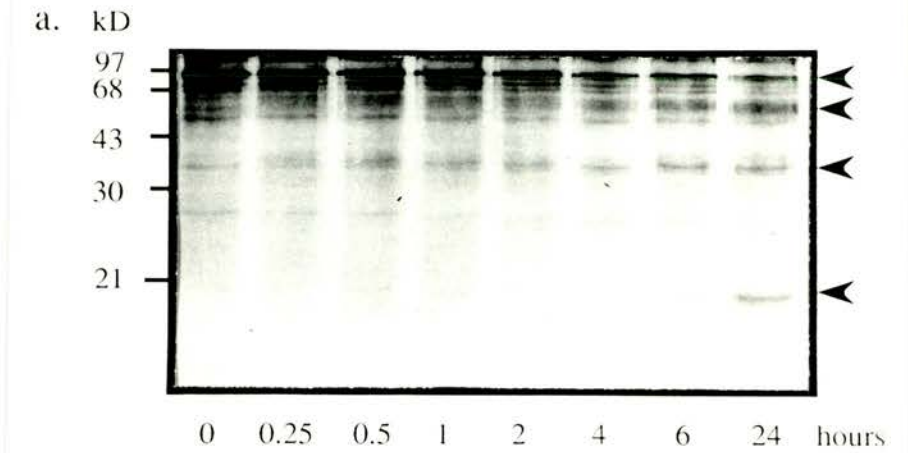
Cleavage of IL-1 Fusion Proteins with Factor Xa Protease

Vlps were digested with 1:100 (w/w) FXa in the presence of 0.02% CHAPS and 0.02% DOC. Aliquots were removed at intervals and the reaction stopped by boiling in SDS-PAGE gel loading mix. Samples were run on 15% SDS-PAGE gels.

(a) IL-1αp:p1 vlp cleavage products stained with Coomassie blue.
(b) IL-1βm:p1 vlp cleavage products stained with silver.

The positions of fusion proteins, p1, initial IL-1 cleavage product and final IL-1 cleavage product are indicated by arrows. Lane 9(b), vlps incubated without FXa, shows that the band appearing at >20kDa under these conditions is a p1 degradation product. This band is not seen in IL-1α cleavage products.

FIGURE 4.9


a.
kD
97
68
43
30
21
0
0.25
0.5
1
2
4
6
24
hours

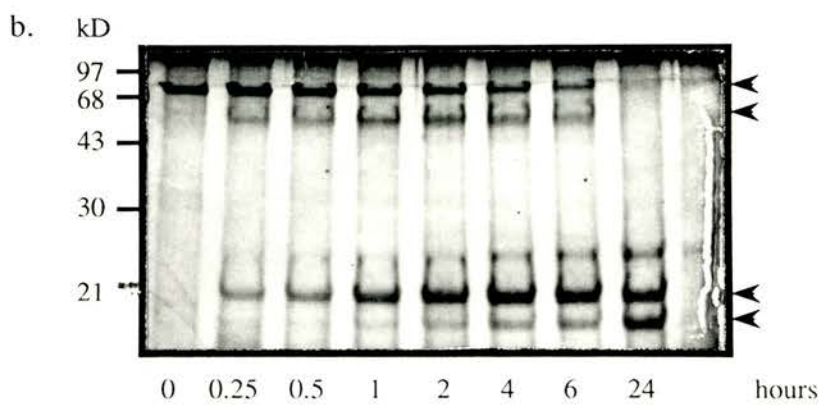

b.
kD
97
68
43
30
21
0
0.25
0.5
1
2
4
6
24
hours

FIGURE 4.10

Recombinant IL-1α Proteins

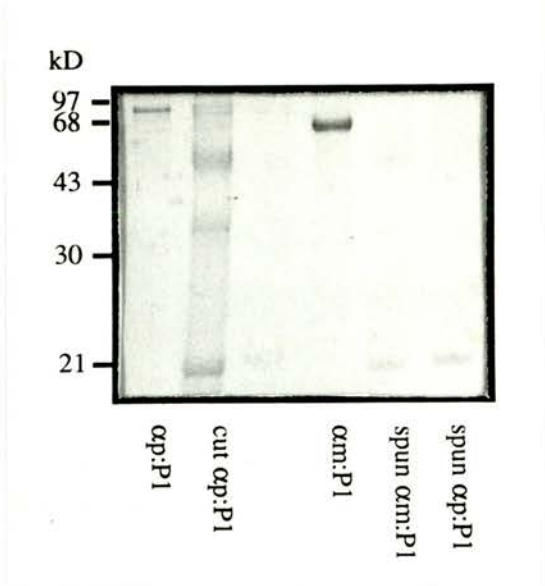


Figure 4.10
IL-1α preparations at various stages electrophoresed through 15% SDS-PAGE gels and stained with coomassie blue. Lanes 3 (not labelled) and 6 are two different loadings of the same sample.

FIGURE 4.11

Purities of Recombinant IL-1 Proteins

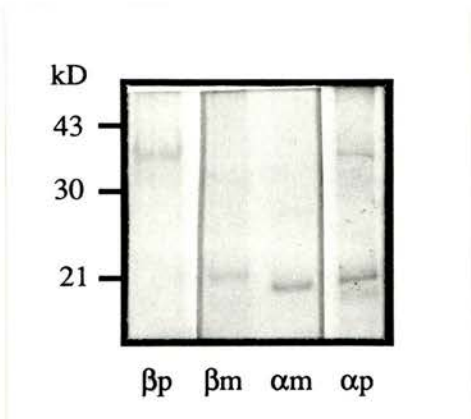


Figure 4.11
Coomassie blue stained 15% SDS-PAGE gel of recombinant IL-1 protein samples after removal of p1 vlps by centrifugation.

4.5 Biological Activity of Recombinant IL-1s

4.5.1 *In vitro* Stimulation of Thymocyte Proliferation by Ovine rIL-1

Figure 4.12.a shows the proliferative response of fresh ovine thymocytes to mature rIL-1α (αm), mature rIL-1β (βm), and rIL-1β preprotein (βp) in the presence of 9μg/ml PHA as co-mitogen in the standard IL-1 assay. Using the accepted unit of activity, (one unit being that amount of protein which yields half maximal counts per minute of incorporated ^{3}H-Thymidine), one unit of ovine αm is ≤0.04ng (≥2.5 x 10^{7} U/mg), one unit of βm is ≤0.8ng (≥1.25 x 10^{6} U/mg). The proportion of upper and lower bands in βm sample preparations did not affect the activity units obtained. One unit of βp is ~3.5ng (2.9 x 10^{5} U/mg) but the level of incorporated counts is 35-40% of the incorporation induced by βm. Neither uncut IL-1:p1 vlps nor a 'FXa cut' p1 protein control (treated exactly as the IL-1:p1 vlps and added to the assay at concentrations up to a hundred-fold greater than those estimated to be in the IL-1 samples) induce thymocyte proliferation. There is some variation in proliferative response from thymus to thymus with respect to both total cpm incorporated and background cpm but the concentrations yielding 1 unit of ovine or porcine IL-1 activity did not vary to any great extent across these assays. The activity of purified natural porcine IL-1β (a gift from Dr. J. Saklatvala, Strangeways Research Laboratory, Cambridge) was assayed for comparison. Using the unit definition of activity, porcine IL-1β, at 1unit = 0.2-0.5ng, is slightly more active than ovine βm and less active than ovine αm. The proliferative response as measured by total incorporation of ^{3}H-T in a number of assays was however about half that induced by either ovine rIL-1 preparation.

About 50 fold higher amounts of αm and 2.5 fold higher βm were required to stimulate murine thymocytes to proliferate under the same assay conditions as used for ovine thymocytes (Fig 4.12.b).

The activity of a preparations containing the truncated form of IL-1αp was compared to IL-1αm activity in an assay shown in Fig.4.13. The unit activities and initial slopes of the curves are similar, but IL-1αp appears to yield a pronounced biphasic curve.

Ovine rIL-1 samples assayed up to 5 weeks after cutting the vlps showed no decrease in their unit activity if stored at 4°C. Aliquots of samples stored at -20°C showed some loss of activity on freezing. Pre-incubation at 37°C for as little as 1 hour caused a large loss of βm activity. (Figure 4.14). IL-1α activity was similarly reduced by freezing (data not shown).

Figure 4.12

Thymocyte Proliferation as a Measure of rIL-1 Activity

a. Stimulation of Ovine Thymocyte Proliferation by IL-1

1 x 10^6 freshly isolated thymocytes were cultured with IL-1 in RPMI;10%FCS;50mM β-ME containing 9mg/ml PHA as a co-mitogen, for 48 hours at 37°C, then pulsed with 1μCi ^{3}H-Thymidine for 24 hours. Cells were harvested onto fibre discs and incorporated radioactivity measured.

Proliferation profiles of ovine thymocytes stimulated by purified recombinant ovine IL-1αm, IL-1βp and IL-1βm expressed in the Ty system are shown. Purified natural porcine IL-1β is included as a contol.
A p1 vlp preparation mock-digested with FXa and treated in exactly the same way as the rIL-1 preparations was included as a negative control. p1 concentrations were matched to those in the IL-1 samples.

b. Stimulation of Murine Thymocyte Proliferation by Ovine IL-1

Fresh murine thymocytes were treated as above.

FIGURE 4.12 a

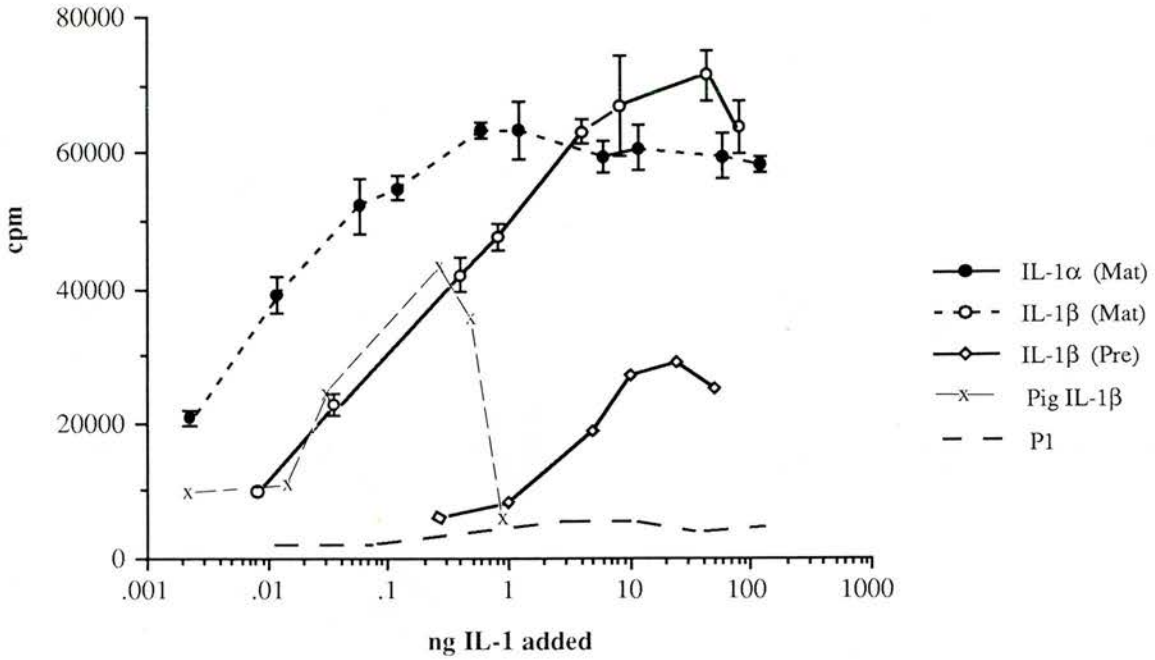


FIGURE 4.12 b

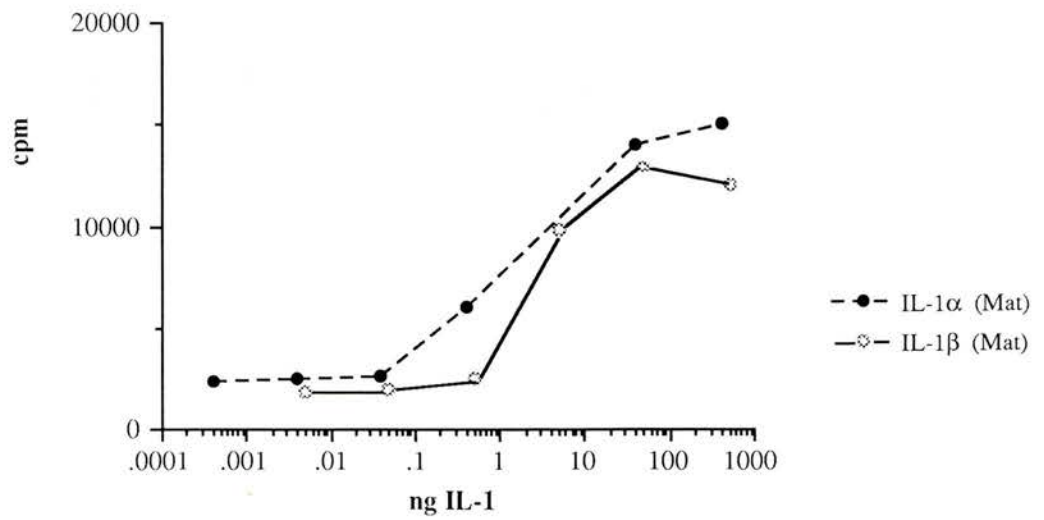


Figure 4.13

Thymocyte proliferation was assayed as described for Figure 4.12.

The activity of a freshly purified IL-1αp preparation which contained the truncated FXa digested form was compared with a fresh IL-1αm preparation. Mock-digested p1 was included in the assay as a control.

In the assay shown, the background counts with medium alone were unusually high (~25000cpm).

Figure 4.14

Thymocyte proliferation was assayed as described for Figure 4.12.

The stimulating activity of aliquots of purified IL-1βm stored either at 4°C in PBS or at -70°C in PBS containing 0.1% bovine serum albumin were compared. The effect of incubating IL-1βm for 1 hour at 37°C prior to adding to the cells was also tested.

FIGURE 4.13

Comparison of rIL-1αm and FXa truncated rIL-1αp Stimulation of Ovine Thymocyte Proliferation

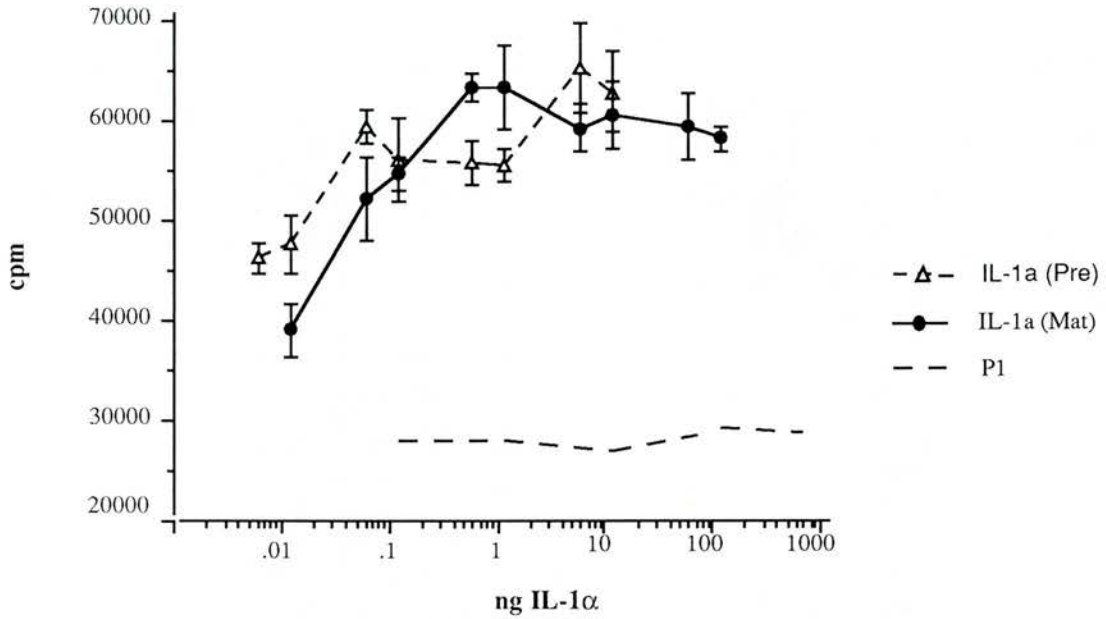
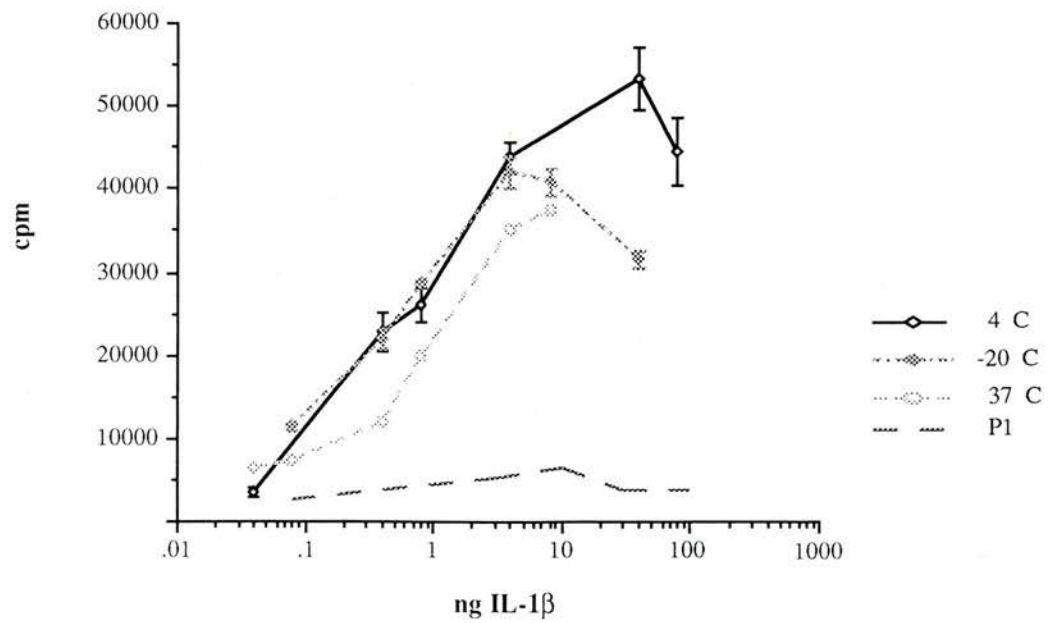


FIGURE 4.14

Effect of rIL-1β Storage Temperature on Thymocyte Stimulation

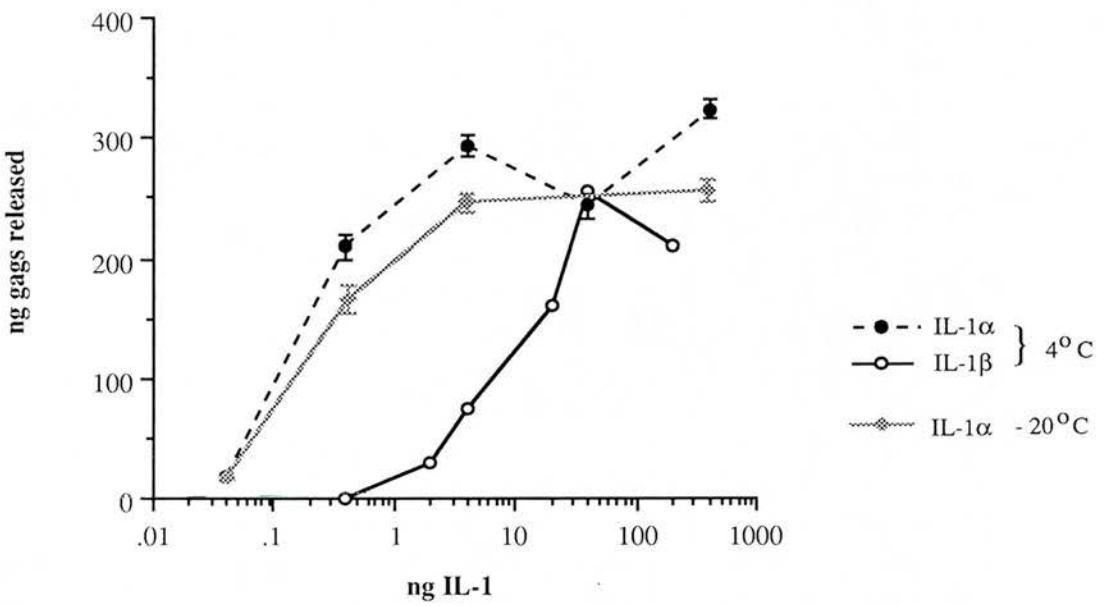


4.5.2 *In vitro* Induction of Cartilage Degradation by Ovine rIL-1

The rIL-1s were assayed for their action in triggering metalloprotease activity with resultant release of glycosylaminoglycans from ovine xiphoid cartilage (Figure 4.15). The activities of both mature proteins were about 10 fold less in this assay than in the ovine thymocyte assay, one unit of αm being ~200pg (5 x 10^6 units/mg) and of βm being ~10ng (1 x 10^5 units/mg). Storage at -20°C also reduced IL-1 activity in this assay, as shown by the curve obtained with IL-1α. IL-1β activity was similarly affected (data not shown). As in the thymocyte assay, none of the controls showed any positive effect in this assay. Porcine IL-1β activity was not compared in this assay due to shortage of material.

FIGURE 4.15

Ovine rIL-1 Induced Degradation of Ovine Xiphoid Cartilage



1 x 2mm discs cut from fresh ovine xiphoid cartilage were pre-incubated for at 37°C for 40 hours in DME medium plus 5% fetal calf serum. IL-1 in fresh medium was added and the discs cultured a further 30 hours. Release of glycosylaminoglycans was measured by adding dimethylene blue dye in formate buffer to aliquots of supernatant. The amount of gags present was determined by comparing OD_{535nm} readings with a standard curve constructed with fresh chondroitin sulphate solutions.

Release of gags by the action of IL-1α and IL-1β stored at 4°C as well as by IL-1α stored at -20°C is shown. IL-1β activity is similarly reduced by -20°C storage (not shown).

Endotoxin levels in all samples were low and did not affect any of the assays descibed above.

4.6 Storage of Fusion Proteins

The fusion proteins are stable for months if stored at 4°C in at least 25% sucrose. If stored in buffer or low sucrose concentrations, the fusion proteins start to degrade within 3 - 4 weeks even in the presence of 0.1% azide. Samples were therefore dialysed into Factor Xa buffer only when required for cleavage. No loss of IL-1 activity occurred under these conditions. Freezing of vlps at either -20°C or -70°C tends to cause clumping with subsequent reduction in both IL-1 yield and activity and is best avoided.

4.7 Polyclonal Antisera to rOvIL-1

Monoclonal antibodies to IL-1α and IL-1β are currently under production. Only polyclonal antisera are dealt with here.

4.7.1 Rabbit Anti-IL-1βm

Rabbits were injected with either uncut βm:p1 vlps or purified IL-1βm samples in which p1 protein concentrations were below the limits of detection by either by coomassie blue or silver staining of SDS-PAGE gels. In both cases anti-IL-1 responses were overshadowed by a strong response to the p1 protein, as demonstrated by a Coomassie stained gel and Western blot of FXa cleaved IL-1β samples from which vlps have not been removed (Fig. 4.16). The polyclonal rabbit antiserum raised against βm, and used at a dilution of 1/50, also cross-reacted with the proprotein, as indicated by arrows. There is no cross-reactivity of the anti-βm antiserum with αm (data not shown). Polyclonal rabbit anti-p1 antiserum, raised against a p1 vlp preparation, does not cross-react with any of the IL-1 proteins on Western blots (see Fig. 4.9 for an example of a cut βm:p1 vlp preparation blotted against anti-p1, in which only the p1 band is positive).

The anti-βm antiserum shows weak neutralisation of IL-1βm activity in thymocyte assays (Fig 4.17). IL-1α activity was not affected by this antiserum. Polyclonal rabbit anti-p1 antiserum used at dilutions of 1/10, 1/100 and 1/1000, did not inhibit IL-1 activity in the assay.

4.7.2 Rabbit Anti-IL-1αm

An initial bolus of 10μg αm in complete Freunds' adjuvant had no adverse effect on the two rabbits injected but a secondary boost of 4μg αm in incomplete Freunds' adjuvant resulted in death after 24-36 hours. I have, to date, no available anti-ovine rIL-1α polyclonal antiserum.

Figure 4.16

Reactivity of Polyclonal Rabbit anti-IL-1βm Antiserum as Visualised on Western Blots

A Coomassie blue stained 15% SDS-PAGE gel shows the positions of uncut IL-1β vlps and IL-1β proteins in cleaved samples from which p1 has not been removed. A companion gel blotted with polyclonal rabbit anti-βm at a 1/50 dilution shows positively reacting bands. The positions of βm and βp on both gels are indicated.

Lane 1, markers which apply to both coomassie stained gel and blot; Lanes 2 - 5, Coomassie stained gel, Lanes 6 - 9, Western blot with anti-βm. Bands containing p1 protein are overexposed in order to visualise the specific IL-1β bands.

Lane 2, uncut βp:p1 vlps; Lane 3, FXa cut βp:p1; Lane 4, uncut βm:p1 vlps; Lane 5, FXa cut βm:p1 with p1 removed by centrifugation.
Lane 6, uncut βm:p1 vlps; Lane 7, Fxa cut βm:p1 (with p1 still present); Lane 8, FXa cut βp:p1; Lane 9, uncut βp:p1 vlps.

Figure 4.17

Neutralisation of IL-1βm Induced Thymocyte Proliferation by a Rabbit Polyclonal anti-βm Antiserum

IL-1 samples were pre-incubated for 1 hour at 37°C with (i) no antiserum; (ii) normal rabbit serum; (iii) polyclonal rabbit anti-βm at 1/10, 1/20 and 1/50 dilutions; (iv) polyclonal rabbit anti-p1 at 1/10 dilution. As controls, medium was incubated with or without the antisera. Pre-incubated samples were assayed with fresh thymocytes as described for Fig. 4.12.

For the sake of clarity standard deviations have not been inserted but none were >5%.

FIGURE 4.16

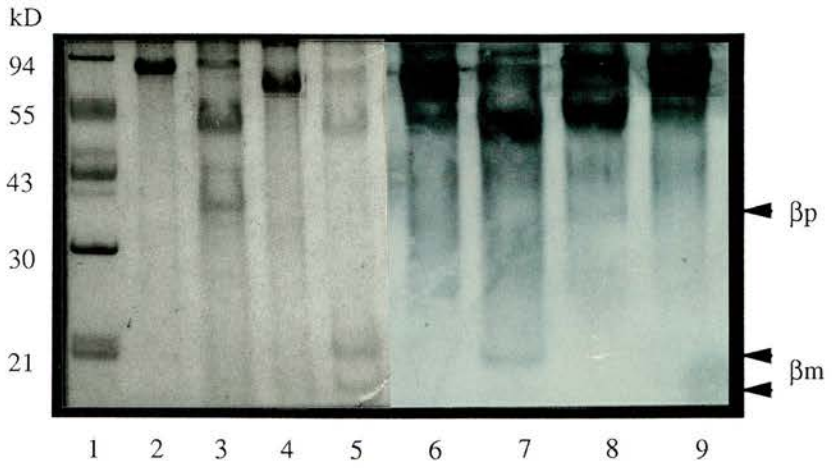
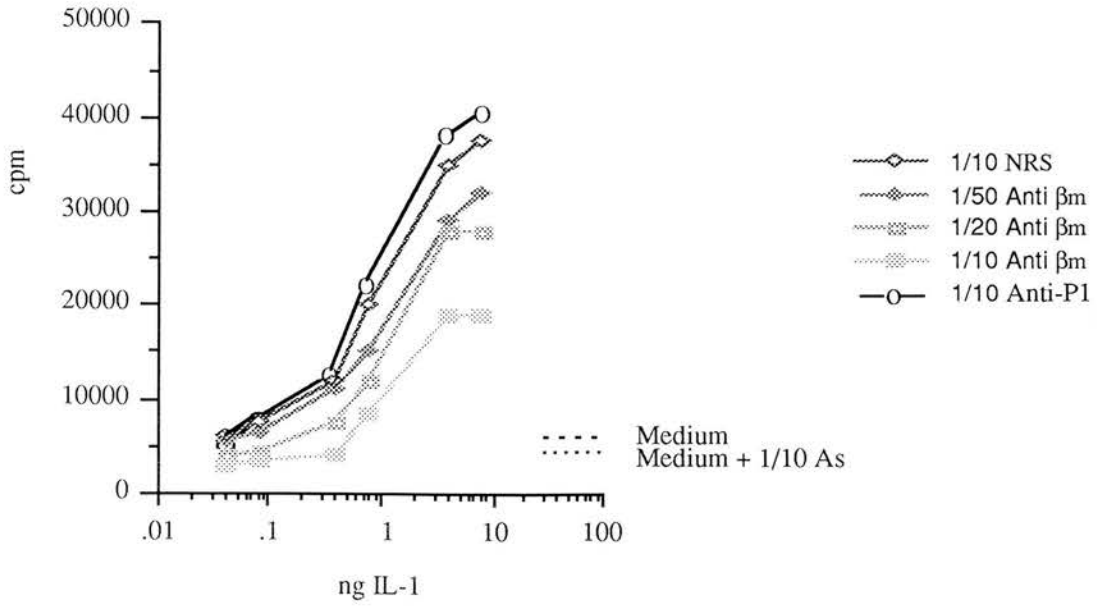


FIGURE 4.17



4.8 HPLC Purification of IL-1α and IL-1β Mature Proteins

It has been demonstrated in Section 4.5 that the small amounts of contaminating proteins in the rIL-1 preparations do not interfere with the biological activity of the proteins. However, receptor binding studies with either iodinated or biotinylated material require rIL-1 samples which are as pure as possible. In addition, because of the antiserum data obtained thus far, it seemed advisable to use HPLC purified material for the generation of both monclonal antibodies and reasonable titre polyclonal anti-IL-1 antisera.

Samples of αm and βm from which p1 had been removed, were further purified by HPLC using a DEAE column with a 40ml 0-500mM NaCl gradient in either 20mM Tris pH8.0. The βm sample chosen for HPLC purification contained minimal detectable amounts of the truncated form. Because of decreased protein concentrations due to spinning out p1 vlps from cleaved preparations, samples were subsequently purified without first removing the p1 and this modification proved to be successful.

0.5ml fractions were collected and 50-100µl aliquots of selected fractions were acetone precipitated and visualised on SDS-PAGE gels. Fig. 4.18 shows pre and post purification material. αm eluted from 200-250mM NaCl and βm from 63-125mM NaCl.

Assay by thymocyte proliferation showed that the fractions containing bands of the correct size were also active at the expected level (Fig. 4.19).

The concentration of pooled αm fractions after HPLC was ~20µg/ml, and of pooled βm was ~1µg/ml. These samples were used for biotinylation and iodination (see Chapter 5).

FIGURE 4.18

HPLC Purification of IL-1αm and IL-1βm

a. Coomassie blue stained 15% SDS-PAGE gel of FXa cut IL-1αm vlps.
Lane 1, Pre-HPLC;
Lane 2, Post purification by ion-exchange HPLC. Acetone precipitated aliquot of a column fraction containing IL-1αm.

b. Silver stained 15% SDS-PAGE gel of IL-1βm.
Lane 1, Pre-HPLC;
Lane 2, Acetone precipitated aliquots from sequential HPLC fractions containing purified IL-1βm.
The mark across the gel at ~50Kd is a staining artefact which appears on all silver stained SDS-PAGE gels.

c. Pooled purified HPLC preparations were dialysed into PBS. Aliquots of these were acetone precipitated, electrophoresed through 15% SDS-PAGE gels and stained with Coomassie blue. These samples were used for labelling purposes (see Chapter 5, Sect. 5.1).
Lane 1, IL-1αm;
Lane 2, IL-1βm.

FIGURE 4.19

Biological Activity of HPLC Purified IL-1αm and IL-1βm

The activity of pre-HPLC and post HPLC purified IL-1 (samples shown in Fig. 4.18.c above) was determined in the standard thymocyte co-mitogen assay as per Fig. 4.12. Mock-digested p1 was included as a control.

A. IL-1αm

B. IL-1βm

FIGURE 4.18

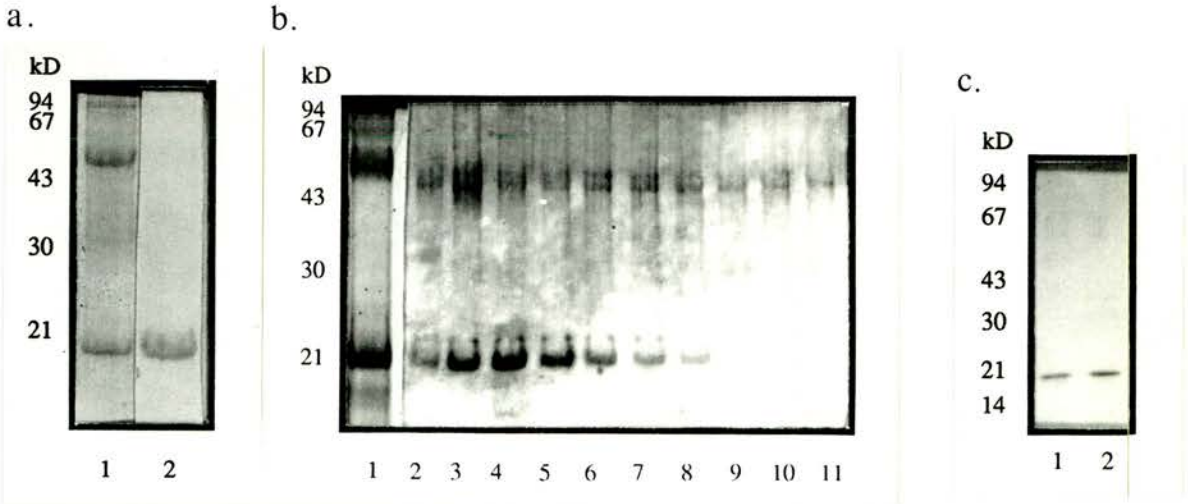
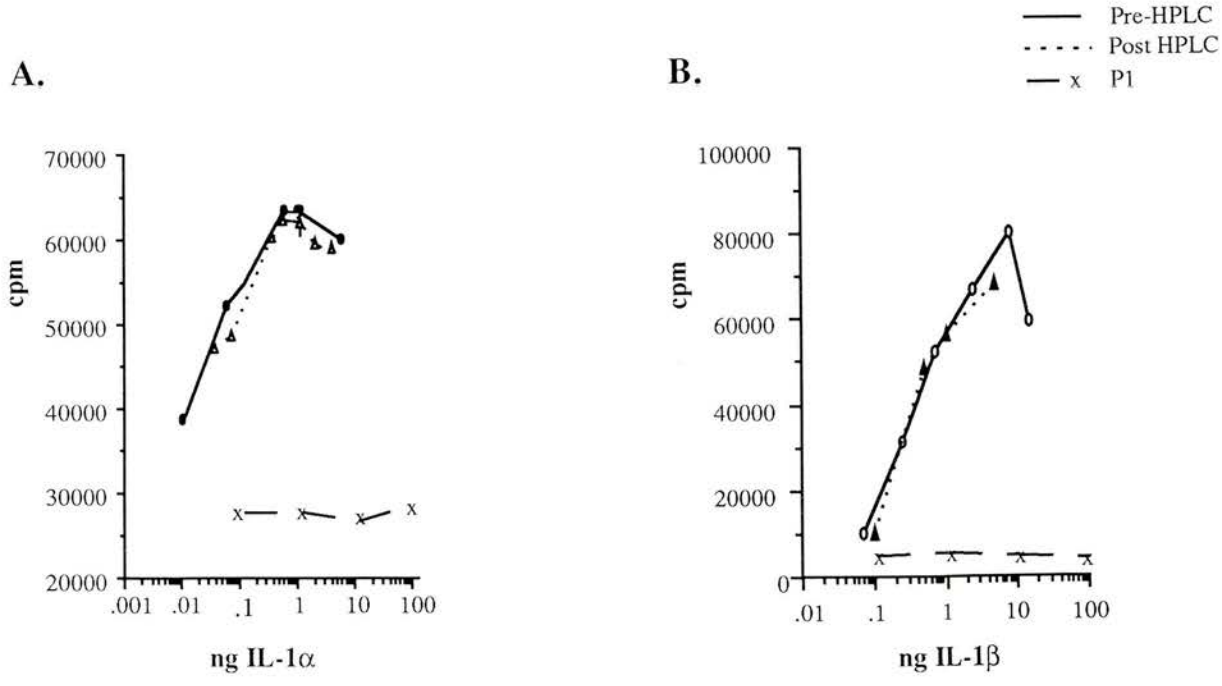


FIGURE 4.19



DISCUSSION

Although the Ty-vlp system had not previously been used for cytokine expression, the advantageous features displayed on expression of viral proteins appeared to fulfil the requirements for recombinant ovine IL-1. From the experience of expressing IL-1 in the Ty-vlp system it has become apparent that these features do not hold universally. Biologically active ovine IL-1α and IL-1β were produced but the protein yields were lower than expected, Factor Xa cleavage of the vlps yielded unexpected products and the immunogenicity of the fusion proteins was not satisfactory. The reasons for this and the consequences are discussed below.

Of the four IL-1 fusion proteins, the IL-1βm vlp yields (3.7mg/l) were lowest and IL-1αm vlp yields highest (8.9mg/l). Yields of the two proprotein vlps were intermediate (5.3-6.2mg/l). In addition, although the initial protocol recommended 24 hours induction, continued induction for longer than 36 hours increased IL-1α vlp yields, αp:p1 more so than αm:p1. 66 hours induction was required to produce equivalent yields of βp:p1 vlps but decreased βm:p1 vlp yields. By comparison, yields of constitutively expressed p1 vlps were 20mg/l after 24 hour culture. As all these vlps were synthesised from the same promoter and the numbers of cells in the 1Am and 1Bm cultures were roughly equivalent, these differences could be indicative of IL-1 vlp stability differences within the yeasts. Evidence of some *in vitro* βm:p1 instability is seen during 4°C storage of vlps, βm vlps tending to start degrading in advance of the others if stored in buffer as opposed to sucrose. Incubation at 25°C can also cause non-specific degradation of βm vlps.

In our department, the Ty system has now been used for the production of a number of recombinant proteins including TNFα, IL-2, IL-2R, IL-6 and maedi-visna retroviral (MVV) proteins. Data is emerging that not all proteins can be expressed efficiently as vlps but not enough data is yet available to evaluate the reasons for these differences.

Vlps form in a range of sizes, as evidenced by sucrose gradient purification and electron microscopy. Individual vlp structures appear to vary. p1 vlps show regular morphology whereas a large proportion of the IL-1:p1 vlps (both IL-1α and IL-1β) show irregularities which are independent of vlp size. These irregularities presumably result from three-dimensional restraints induced by the recombinant protein. Distorted morphology has been similarly reported for HIV-1 p24 and MVV p25 vlps (Gilmour et al., 1989; Reyburn et al., 1992).

Ovine IL-1αm was synthesised with N-terminal Ser-120 following bovine IL-1α data (Maliszewski et al., 1988). This protein was active in the expected range (see below). Ser-113 is most often cited as the N-terminal of human or murine IL-1αm. Active proteins or subpeptides with other N-termini are however routinely detected in biological fluids (Cannon and Dinarello, 1985). A number of enzymes have been implicated in the processing of the IL-1α preprotein. A calcium activated neutral protease, calpain, digests the protein at Ser-113 (Kobayashi et al., 1990). Trypsin and chymotrypsin (cut sites *Arg ^{v}Ser* and *Phe ^{v}Leu* respectively) as well as serine proteases have all been proposed as IL-1α processors (Lomedico et al., 1984, Cameron et al., 1986, Cannon and Dinarello, 1985). Of interest is that the putative sequence at which Factor Xa is cutting the αp:p1 vlps is *IleuLysPro**Arg^{v}Ser***, Gln replaces Lys in the mouse but otherwise this sequence is conserved across all other species so far sequenced (see Chapter 6 for full sequence comparisons). Jones and Geczy (1990) found that Factor Xa and thrombin greatly increased IL-1 activity in macrophages stimulated by suboptimal concentrations of LPS. It was proposed that this was via FXa conversion of prothrombin to thrombin. The Factor Xa cleavage pattern of purified αp:p1 vlps has raised the interesting possibility that FXa could be acting directly on IL-1α.

IL-1 moves anomalously in SDS-PAGE gels which may be due to the highly charged nature of the proteins. This phenomenon has been reported for various polypeptides (Marnell and Summers, 1984). Bovine and human rIL-1 (Maliszewski et al., 1988; March et al., 1985), as well as ovine IL-1 expressed in an *E. coli* system (P. Wood, personal communication, 1993), behave in a similar fashion.

It is difficult to quantify differences in IL-1 activity as measured in biological assays. From the thymocyte proliferation curves obtained, it is obvious that both total ^{3}H-T incorporation and unit activity values have a bearing. For example, the initial slopes of ovine and porcine IL-1β induced proliferation curves are superimposable but the extent of proliferation, as measured by maximum cpm incorporated, is about 1.5 times greater with the ovine material. The unit activities of these two proteins, however, indicate that porcine IL-1β is slightly more active in this system than ovine. Similarly the proliferation curve obtained from rIL-1 which had been stored at -70°C showed the same initial slope but with reduced maximum incorporation. In the following discussion, stated IL-1 activities are based on the generally accepted definition of IL-1 unit activity which is:- that amount of IL-1 which will induce half-maximal proliferation.

The activities of ovine rIL-1s in thymoctes assays, ~$2.5x10^7$ units/mg IL-1α and ≥$1.25x10^6$ units/mg IL-1β, compare favourably with reported thymocyte activities of human and murine rIL-1s (Lomedico et al., 1984; Gubler and Hoffman, 1983; Huang et al., 1988; Tocci et al., 1987) and purified natural bovine IL-1 (Lederer and Czuprynski, 1989b), ie. Ranging from 1 x 10^6 to 5 x 10^7 units/mg for IL-1α and 1 x 10^6 to 5 x 10^6 units/mg for IL-1β. The reported activities of human rIL-1 expressed in *E. coli* are only very slightly lower than those of murine rIL-1 from *E. coli,* when assayed on fresh mouse thymocytes or on murine cell lines, a reflection of the close homology of the human and murine proteins. Results obtained from ovine rIL-1 stimulation of ovine and murine thymocytes show a preference for the homologous cells, a phenomenon already described with respect to the activity of natural purified bovine IL-1 (Lederer and Czuprynski, 1989b), recombinant bovine IL-1 (Maliszewski et al., 1988) and recombinant human IL-1 (Thieme et al., 1987). The human rIL-1 did not stimulate ovine or bovine thymocytes to proliferate. From preliminary work for the study being reported here, there was little evidence of commercially produced anti-hIL-1 antisera having much neutralising activity against ovine macrophage supernatants in the thymocyte assay (data not shown). This preference for homologous cells needs to be taken into account when addressing the biological activity of IL-1, especially as the species specificity appears to depend on which IL-1-mediated activity is being examined. For example, human endothelial cells can be induced by both human and murine IL-1α to produce platelet-activating factor whereas only the human IL-1α induces secretion of PGI_2 in these cells (Dejana et al., 1987).

Just prior to submitting this thesis it was reported that the activity of ovine IL-1βm expressed in E. *coli* and assayed with ovine thymocytes was >10^6 units/mg (P. Wood, personal communication, 1993), similar to the activity of the Ty-expressed ovine IL-1βm reported here.

The activities of both IL-1α and IL-1β are about ten times lower in the cartilage assay than in the thymocyte assay. Freezing rIL-1 at -20°C has also been shown to result in a loss of ovine rIL-1 activity, thymocyte proliferation being less affected than cartilage degradation.

None of the IL-1:p1 vlps show any biological activity. Presumably residues required for receptor binding are not accessible.

Despite the use of fresh tissue, the reproducibility of the thymocyte assay was good. IL-1αm activity ranged from $2.5x10^7$ to $5x10^7$ units/mg and IL-1βm activity from $1.25x10^6$ to $3x10^6$ units/mg. IL-1α and IL-1β stimulation of thymocytes did however produce different proliferation profiles. When assayed

concurrently, the amount of IL-1αm required to induce half-maximal proliferation was about 20 times less than IL-1βm but the maximum cpm of incorporated ^{3}H-thymidine (^{3}H-T) induced by IL-1β was greater. The initial slopes of the proliferation curves were similar for all the recombinant ovine IL-1s and as well as for natural purified porcine IL-1β (see Fig. 4.12.a). Ovine IL-1βp and IL-1βm induced ^{3}H-T incorporation both reached maximal levels at 20-30ng then decreased dramatically with increasing IL-1 concentration. Porcine IL-1β produced a similar profile but with a much lower amplitude of incorporated counts. In contrast, IL-1α induced incorporation seemed to plateau at ≥1ng IL-1.

The data obtained for IL-1βm activity suggests that truncated 18kDa form is active because the activity detected in thymocyte and cartilage assays seemed to be independent of the proportions of 20kDa and 18kDa forms in the preparations. Antibody studies and mutational analyses reported in the literature are conflicting as to the essential requirements for IL-1β activity and there is little data on the importance of C-terminal amino acids. One study suggests that amino acids 163-171 and 187-204 are the essential domains for IL-1β activity (Boraschi and Tagliabue, 1990), each being important for different functions, but another study has suggested that amino acids up to 259 are essential for activity (Mosley et al., 1987a). Yet others have reported a core peptide which retains biological activity (Schmidt and Bomford, 1991). It seems to be clear that even point mutations at distinct locations can uncouple IL-1 activities (see Chapter 1 Sect. 1.11.1.6). Whether the loss of amino acids 244-266 from IL-1β has any biological relevance has yet to be determined. Residue 245 is fully conserved across IL-1α, IL-1β and the IL-1 receptor antagonist which may indicate a structural or receptor binding rather than signal transducing function. From the data obtained with ovine IL-1β, it appears that loss of this residue does not adversely affect IL-1β activity, at least with respect to ovine thymocyte proliferation or cartilage degradation. Both these cell types possess the type I IL-1 receptor which has a greater preference for IL-1α than IL-1β (see Chapter 1 Sect. 1.11.1.3). It may be that the truncated form would not be as active on cells possessing the type II receptor which have a greater preference for IL-1β than IL-1α. IL-1 structure/function relationships will be discussed in Chapter 6.

Human IL-1β possesses a glycosylation site at residues 123-125 in the mature protein. Glycosylated rhIL-1β mature protein has been produced in yeast and CHO cells but only by use of fused hybrid secretory sequences (Casagli et al., 1989; Pecceu et al., 1991). This protein is inactive until deglycosylated. It is possible that human IL-1β could be glycosylated *in vivo* although there is no evidence for this. Glycosylation may inhibit receptor binding as three residues

(Arg-120, Leu-122, Glu-128) of the seven implicated in receptor binding (Labriola-Tompkins et al., 1991) are in very close proximity to this site. The ovine rIL-1s are not expressed as glycosylated proteins in the yeast Ty system.

The choice of vector, (including promoter and enhancer) and expression system affect the production and activities of recombinant proteins. This is the case with glycosylated mature hIL-1β as mentioned above and is also clearly seen with respect to IL-1β proprotein, IL-1βp, which until recently has been thought to be biologically inactive *in vivo*. Ovine IL-1βp, synthesised from the PAL promoter in yeast, was bioactive although the unit activity was about five times less than IL-1βm and the amplitude of ^{3}H-T incorporated in the thymocyte assay was about 30% of that after induction by IL-1βm. *E. coli* has been reported not to produce active IL-1βp but there are three reports of the recombinant protein having some activity when expressed in other systems. Two studies using mammalian COS-7 cells and one using *in vitro* translation have produced recombinant IL-1βp which is active on the murine D10.G4.1 cell line and on fresh thymocytes.

The one COS-7 cell study produced active human IL-1βp under full control of SV40 sequences (Rosenwasser et al., 1986). The relative activities of the hIL-1βp and hIL-1βm proteins from this system were similar to those found for the ovine proteins in the thymocyte assay.

Interestingly, the other COS-7 cell study, by Andrews et al, (1992), concerned ovine IL-1βp expressed from a vector containing the SV40 origin of replication, enhancer and polyA signal, and under control of a human metallothionine promoter. In this system, deletion of the 3' untranslated sequence which is supposed to confer instability on the mRNA, resulted in greatly increased yields of active IL-1α and IL-1β preproteins. Figures are not quoted for protein yields or purities and the activities are quoted as raw counts not units. It is therefore impossible to relate these quantitatively to the ovine IL-1 activities which I found.

In the third report, Jobling et al. (1988), synthesised biologically active hIL-1βp in the rabbit reticulocyte translation system using bacteriophage SP6 polymerase (Melton et al., 1984; Krieg and Melton, 1984). The protein yield was significantly increased by replacing the cognate IL-1β leader sequence with the 37 nucleotide plant viral leader, alfalfa mosaic virus RNA 4. Only by enhancing mRNA translational efficiency, using the chimeric mRNA, was sufficient protein produced to stimulate thymidine incorporation. The specific activity of IL-1β proprotein was 6.8 x 10^{5} units/mg in the D10.G4.1 assay and the specific activity of IL-1β mature protein, synthesised and assayed in an identical way,

was 1.7 x 10^8 units/mg, similar to that reported for IL-1βm synthesised in *E. coli* and assayed with D10.G4.1 cells. (March et al., 1985). Generally, human and murine IL-1β D10.G4.1 activities seem to be 50 - 200 times greater than thymocyte activities (Auron et al., 1984; March et al., 1985; Jobling et al., 1988). On this basis, ovine IL-1βm and IL-1βp activities in the ovine thymocyte assay (≥1.25 x 10^6 units/mg and ~2.9 x 10^5 units/mg respectively) were both roughly comparable to the above.

From the above, transcriptional and translational control of rIL-1 production both seem to be important with respect to its activity. Posttranslational modification may also be of importance. The importance, therefore, of producing recombinant proteins with characteristics as close as possible to the natural protein, is obvious.

Immunisation of rabbits with IL-1βm vlps produced a minor anti-IL-1β and major anti-p1 response which may have been a consequence of the irregular shape of the vlp. p1 has been found to be extremely immunogenic (Kingsman and Kingsman, 1988) and it may be that presentation of a recombinant protein in the form of a vlp is only advantageous if the vlps are completely regular and the p1 protein presumably not accessible. Purified rIL-1βm from which the cleaved p1 had been spun out and was not detectable by coomassie staining was subsequently used for immunisation. Again a strong anti-p1 response was obtained. There is no amino acid homology between p1 (Mellor et al., 1985) and IL-1 and neither does anti-p1 antiserum react with either IL-1 protein on Western blots. IL-1 has been advocated for use as an adjuvant (Tagliabue et al., 1991) and it is possible that IL-1β could be acting as an adjuvant for the p1 response in this case. In view of the 76% similarity between the rabbit and sheep mature IL-1β proteins, there may also be limited 'foreign' IL-1 sequence for the rabbit to recognise, resulting in a low-grade response to the ovine IL-1.

It has been presumed in the literature that a conformational change, resulting from the enzymatic removal of the N-terminal portion of IL-1βp is required for the generation of biologically active IL-1βm *in vivo* (Mosley et al., 1987a). The rabbit anti-ovine-IL-1βm polyclonal antiserum cross-reacted with ovine IL-1βp on Western blots indicating that at least some of the epitopes are similarly exposed in both proteins. There is a possibility that the folding pattern of recombinant IL-1βp protein may not be identical to that of the natural molecule. If so, this may also have some bearing on the activity detected although the antiserum was not tested for neutralisation of βp activity. There are no reports in the literature of antisera to IL-1β cross-reacting with IL-1α and*vv*. Polyclonal anti-ovine-IL-1βm also did not cross-react with ovine IL-1αm on Western blots.

In conclusion, biologically active preprotein and mature forms of ovine IL-1α and IL-1β have been expressed in as recombinant proteins in *Saccharomyces cerevisiae* strain BJ2168 using Ty-vlp expression vectors. Yields of the purified proteins range from 0.3 to 1mg/l and purities have been estimated at 80-95% prior to HPLC purification.

CHAPTER 5

IL-1 RECEPTOR EXPRESSION BY OVINE AFFERENT LYMPH DENDRITIC CELLS

INTRODUCTION

There is currently much interest in the mechanism whereby T cells become sensitised to antigen and on the induction of cytotoxic T cell responses by specialised antigen presenting cells (APC), dendritic cells, macrophages and B cells being considered to be the most potent of these. Accessory cells perform at least two important functions with respect to T cell responses to antigen, (i) to present antigen, usually in association with major histocompatibility (MHC) Class I or Class II glycoproteins, for recognition by clonotypic antigen receptors on T cells; (ii) to activate and induce subsequent proliferation of antigen specific T cells. Generally, antigens taken up by APC, are processed within these cells to small peptides, 8-12 amino acids, which are presented to T cells in association with specialised glycoproteins, the most common being MHC Class I and Class II molecules (Monaco, 1992; Neefjes and Ploegh, 1992). Endogenously derived peptides are presented by Class I molecules and exogenously derived peptides by Class II molecules. A wide variety of MHC Class II-positive cells induce proliferation of previously activated T cells but only dendritic cells have the capacity to cluster with and stimulate proliferation of normal resting T cells (Inaba et al., 1984). Knowledge about the sphere of influence of cytokines on both antigen processing and presentation is assuming increasing importance and it is becoming clear that data from one type of APC can not necessarily be extrapolated to another.

Dendritic Cells

Dendritic cells (DC) form a system of antigen presenting cells which function to initiate several immune responses such as the sensitisation of MHC-restricted T cells, the rejection of organ transplants and the formation of T-dependent antibodies. Immature DC, which are highly specialised to process foreign antigens, reside in non-lymphoid tissues. They migrate via the afferent lymphatics or the blood to the T-dependent areas of lymphoid organs where, as mature interdigitating dendritic cells, they can efficiently stimulate resting antigen-specific T cells. DC do not migrate further and are not found in efferent

lymphatics (Pugh et al., 1983; Knight, 1984; Kraal et al., 1986; Bujdoso et al., 1989; Fossum 1989; Larsen et al., 1990). Follicular dendritic cells which are involved in the regulation of B cell function are derived from a different lineage and are not under consideration here.

Cells with a dendritic, veiled, cell morphology were first descibed in afferent lymph in 1978 by Kelly et al. and have become the subject of increasing interest as potent antigen presenting cells. Following exposure to sensitising chemicals, dendritic cells rapidly accumulate in the draining lymph nodes. A proportion of the DC arriving at the nodes bear significant amounts of antigen and are derived from epidermal Langerhans' cells (LC). LC, immature DC, are relatively inefficient antigen-presenting cells but as they migrate from skin to lymph node they are subject to a phenotypic maturation and the antigen-bearing DC found within the draining node are potent accessory cells which can induce immune responses both *in vivo* and *in vitro* (Steinman, 1991; Fossum, 1991; Romani and Schuler, 1992). 5-10% of the afferent lymph mononuclear cell population consists of DC. Not all of these DC are skin derived, as indicated by the absence, in a small percentage of cells, of CD1a+ and $CD1c^+$ expression, specific markers for LC (Caux et al., 1992).

Earlier controversy as to whether DC are metabolically active and whether they phagocytose and process antigen is now beginning to be resolved. Recent data indicates that LC are initially endocytic but as their antigen presentation potential increases, the cells no longer process antigen. Endocytosis through late endosomes has been shown to be as active in DC as in other antigen presenting cells and chloroquine inhibition of antigen processing has confirmed a central role for acidified endocytotic processing to form peptide-class II MHC complexes for presentation to $CD4^+$ T cells (Stossel et al., 1990; Cohen and Katz, 1992; Hoyne et al., 1993; Levine and Chain, 1992). This is not, however, the complete picture. Membrane peptidase mechanisms are expressed by DC and there is evidence that initial processing may release peptides which are subsequently internalised and processed (Chain et al., 1989; De Bruijn et al., 1992). Immune complexes may also be retained on the surface of some DC for the purposes of antigen transport (Szakal et al., 1989). The expression of Fc receptors, which enhance antigen uptake by B cells and macrophages, does not seem to be a uniform characteristic of DC and may be related to the above. Murine lymph node and spleen (Nussenweig et al., 1981) and human peripheral blood DC (van Voorhis et al., 1982) do not appear to express FcR whereas rat lymph node (Schalke et al., 1985), murine pulmonary tissue (Sertl et al., 1986) and Langerhans cells (Spry et al., 1980) and human afferent lymph do (Witmer-

Pack et al., 1988). FcR$^-$ DC have been implicated as the major antigen presenting cell in the murine thymus (Inaba et al., 1988).

IL-1, Dendritic Cells and the Immune Response

Antigen presentation by dendritic cells requires antigen/Ia (Class II) complex for specific recognition of antigen. Until comparatively recently, not a great deal has been known about the interactions of DC and cytokines but information is accumulating increasingly rapidly. Much of the data currently in the literature has been derived from tissue culture studies using cells derived from skin, thymus, spleen, tonsil and lymph nodes. Some differing DC characteristics have been detected which may be a consequence of localised and specific functional requirements of the DC in the different tissues.

IL-1 mRNA has not been consistently detected in DC populations from lymphoid tissue or peripheral blood (Calder et al., 1992; Hopkins et al., 1990; Koide et al., 1987b, 1988; Vakkila et al., 1990; Waalen et al., 1986) and IL-1 production may depend on the state of differentiation or activation of the cells. DC from inflammatory sites appear to produce large amounts of IL-1 spontaneously while associated peripheral blood DC produce minimal amounts (Waalen et al., 1986). Maturing DC in culture have been found to produce large amounts of IL-1β (Ruppert and Peters, 1991; Heufler et al., 1992).

From much of the published work on DC as antigen presenting cells, an amplifying rather than an initiating role is currently being ascribed to IL-1. It appears that IL-1 is not essential as a second signal for allogeneic T-cell responses, but rather enhances the function of the accessory dendritic cells (Bhardwaj et al., 1989; McKenzie et al., 1989; Vakkila et al., 1990; NaitoK et al., 1989). Rabbit and murine DC function can be amplified by pre-exposure to IL-1 (Kapsenberg et al., 1985; Koide et al., 1987a) and this enhanced function is not blocked by anti-IL-1 (Inaba et al., 1988), suggesting direct action of IL-1 on the DC not the T cell.

GM-CSF is a major macrophage activating factor which, apart from inducing cytokines, also induces macrophage HLA-DR expression (Chantry et al., 1990). It is currently thought that GM-CSF is the most important mediator of the maturation of LC into DC (Heufler et al., 1992). GM-CSF and TNF have now been shown to co-operate in the generation of dendritic Langerhans cells from hematopoetic stem cells (Caux et al., 1992). *In vivo*, IL-1 may synergise with GM-CSF and TNF to initiate mobilisation of Langerhans cells from the epidermis and maturation into lymph borne dendritic cells (Cumberbach and

Kimber, 1992). Whether IL-1 is a requirement for this process is not certain, although there is experimental evidence that injection of human IL-1α into pigs induces transient dose-dependent lymphocyte traffic into the draining lymph nodes which peaks at 4h, earlier than that induced by bovine or human TNFα which peaks at 9-12h (Binns et al., 1992). There are a number of reports of direct augmentation of the DC/Tcell reaction by GM-CSF or GM-CSF plus IL-1 (Heufler et al., 1988; Koide et al., 1988; Wilson et al., 1988). Some authors suggest that IL-1, in contrast to TNF, has no effect on DC viability. TNFα on the other hand does not appear to be involved in either maturation of DC or antigen presentation (Koch et al., 1990; Hosoi et al., 1993).

DC rapidly upregulate adhesion molecules *in vitro* (Teunissen et al., 1990) which is important with respect to the immunogenicity of DC associated with antigen. Recent studies have shown that stratum corneum-derived human IL-1 injected into the skin will upregulate dermal dendritic cell expression of the adhesion molecules ICAM-1 and VCAM-1 (Groves et al., 1992).

Much remains to be learnt about IL-1 involvement both in antigen uptake by DC and in antigen presentation.

Sheep Afferent Lymph Dendritic Cells

Because of the importance of afferent lymph dendritic cells in antigen presentation and their pivotal role in the immune response, the relationship between these cells and IL-1 needs to be established. Any influence of IL-1 on DC could be via either direct or indirect mechanisms. From the data available at the start of this study, it seemed highly likely that DC could possess IL-1 receptors. One of the main reasons for the lack of data on fresh DC is the problem of obtaining sufficient numbers of DC *ex vivo*, especially if working with small animals. Because of the ease of cannulation of its lymphatic ducts, the sheep is ideal for the study of cells in afferent lymph. Using this system, fresh cells can be continuously collected hence obviating cell culture, which could potentially affect the characteristics displayed by the cells. Cannulation also allows detection of early *in vivo* events following antigen challenge of the draining skin area.

DC numbers in normal sheep afferent lymph are $\pm 1 \times 10^5$ DC/ml, comprising 5-8% of the cell population and sufficient for isolation provided the lymph flow rate is reasonable. Study of DC freshly isolated from sheep afferent lymph would be expected to give information on DC characteristics expressed *in vivo*, both in a resting state and after antigen challenge.

Macrophages (Mϕ) are also potent antigen presenting cells but to a much lesser degree than DC. Various differences between cell surface markers expressed by macrophages and DC have been noted on human and murine cells. Very few markers, however, have so far been proposed for specific identification of DC and, because of lack of species cross-reactivity, none of these can be used for definitive identification of sheep DC. As opposed to macrophages, DC are only transiently adherent to glass or plastic, a fact which is utilised for separation of the two populations.

Much work has been done in our department on the characterisation of sheep afferent lymph dendritic cells (Bujdoso et al., 1989, 1990; Hopkins et al., 1989; Harkiss et al., 1990). Sheep afferent DC constitutively express $\pm$ 3 x 10^5 MHC Class II molecules per cell but cells entering the lymph node from a site of secondary antigen challenge express a sixfold higher level than this. The majority of DC express surface immunoglobulin (Ig) of IgM and IgG1 types. At least four subpopulations of dendritic cell enter the lymph node via the afferent lymph; $CD1^+$, $CD1^-$, FcR^+, and FcR^-. It is not yet clear whether these represent distinct cell types or cells in transitional states. Evidence for the latter comes from cultured murine LC which lose expressed FcR as they develop into DC (Witmer-Pack et al., 1988; Romani et al., 1989). Approximately two thirds of sheep afferent lymph DC bind antigen/antibody complexes via Fc receptors. Of these cells, about 43% were shown to be $CD1^+$ and are likely to be LC derived. The complexes may be internalised and processed, or the DC may be acting as carriers presenting antigen in an undegraded form. Class II positive, FcR^+ DC are known to be efficient at presenting antigen to $CD4^+$ T cells and the $CD1^+$, FcR^- dendritic cell has been proposed as the principal presenting cell for ovine γδ T cells via a heat shock protein/CD1 ligand which would obviate the necessity for FcR (Harkiss et al., 1990). Whether there is restriction amongst the lymph DC subsets with respect to antigen presentation has still to be clarified.

Morphologically different DC subpopulations are found in sheep afferent lymph but little is known about their origin and surface antigen expression. In culture, murine DC developing from LC become veiled cells, showing highly characteristic sheet-like cytoplasmic processes and being typically devoid of organelles (Romani et al., 1989). These cultured cells are not necessarily identical to freshly isolated DC. Interdigitating dendritic cells while they are resident in tissues display dendritic processes and contain cell organelles whereas, once isolated, they display veiled cell characteristics. In culture, the dendritic processes seem to be constantly extended and retracted. The sequence of events during *in vivo* differentiation is not known and veiled morphology can

not therefore be assumed to be the final stage of differentiation. LC are generally identified by the presence of Birbeck granules which can be seen in ultrathin sections viewed in the electron microscope (Birbeck et al., 1961), or by membrane ATPase (Juhlin and Shelley, 1977). Maturing murine and human LC in culture may lose these LC specific Birbeck granules but this does not seem to be the case for all LC/DC and can not be used as a reliable indicator of maturation stage (Schuler et al., 1985; Romani et al., 1989; Bucana et al., 1992). Ovine LC do not stain for ATPase but epidermal dendritic cells which contain acetylcholinesterase (AChe) have been identified specifically as being LC (Hollis et al., 1972). Changes in AChe staining with maturation of ovine LC has not been determined but AChe activity might be expected to diminish with decreasing processing potential.

Antigen presentation to T cells by sheep afferent lymph DC is much more efficient during secondary responses to antigen. From data showing increased proliferative responses due to increased uptake of antigen in the presence of specific antibodies, it has been proposed that FcR mediated uptake could represent a mechanism whereby antigen can be concentrated during secondary responses (Harkiss et al., 1990).

The mechanism whereby IL-1 influences the DC has yet to be elucidated. One of the methods by which the response of DC to IL-1 could be studied, is by determining IL-1 receptor expression by DC.

IL-1 Receptors

Two types of cellular IL-1 receptor have so far been characterised and have been described in detail in Chapter 1. Although IL-1α and IL-1β differ markedly in amino acid sequence, the two proteins bind to the same receptors and induce similar biological responses (reviewed in Chapter 1, Sect. 1.11.1.3). The binding is specific and saturable but the relative affinities differ depending on cell type. The two receptors represent different gene products whose extracellular structures are similar but whose intracellular portions are very different and induce different signal transduction pathways (see Sect. 1.11). IL-1 can upregulate both IL-1RI and IL-1RII (Akahoshi et al., 1988b; Chin et al., 1988; Sheih et al., 1990; McMahan et al., 1991; Takii et al., 1992) but has also been reported to downregulate its own receptor on fibroblasts, large granular lymphocytes and some T cell lines (Mizel et al., 1987; Matsushima et al., 1986b; Lacey and Erdmann, 1990), these two different effects possibly being IL-1 concentration dependent (Takii et al., 1992).

Having expressed biologically active recombinant ovine IL-1α and IL-1β, I was in a position to attempt to define more clearly the relationship between afferent lymph DC and IL-1. The study reported below was designed to answer some of the basic questions and has involved demonstrating the presence of IL-1 receptors on afferent lymph DC and determining the effect of localised antigen challenge on the surface expression of these receptors.

Summary of Experimental Strategy:

Sheep prefemoral lymph nodes were excised and the animals primed with 500μg ovalbumin (in complete Freund's adjuvant), injected intradermally at three sites round the lymph node area. After at least eight weeks, the sheep were boosted with 50μg ovalbumin (in PBS) and 6 weeks later the anastamosed pseudoafferent lymph ducts were cannulated. Cannulations were allowed at least three days to stabilise before afferent lymph was collected into heparinised bottles. For studying the effects of secondary antigen challenge, the animals were injected with 50μg ovalbumin a few days after cannulation.

DC enriched and lymphocyte fractions of lymph cells were separately incubated with ovine rIL-1. For determination by Scatchard analysis of numbers of receptors expressed and their dissociation constants, ^{125}I-rIL-1 specifically bound to the cells was counted. The binding of ^{125}I-rIL-1 at an individual cell level was visualised on cytospins exposed with Amersham LM-1 emulsion. Binding of biotinylated rIL-1 was visualised by FACS (Fluorescence Activated Cell Sort) analysis. Ovine alveolar macrophages were used as positive control cells for rIL-1 binding.

The following progression of experiments was employed;

1. Iodination and biotinylation of rIL-1
2. Assessment of biological activity of labelled proteins
3. Identification of macrophages (Mϕ) vs afferent lymph dendritic cells (DC)
4. Detection of IL-1 receptors on alveolar macrophages
5. Detection of IL-1 receptors on resting DC
6. Establishing a binding assay
7. Quantitation of IL-1 receptors on DC and Mϕ
8. Expression of IL-1 receptors on DC and lymphocytes during primary and secondary responses to ovalbumin challenge

5.1 Iodination and Biotinlyation of Recombinant Proteins

Protein iodination and biotinylation occur via different amino acid residues. Iodination involves tyrosine and histidine (Tyr preferentially). Biotinylation involves lysine, asparagine, glutamine and arginine. Table 5.1 shows rIL-1 and p1 residues available for labelling.

TABLE 5.1

Amino Acid Residues Available for Labelling IL-1 and p1 Proteins

a. Residues available for iodination:

	Residues per molecule	Y per µg	Y + H per µg
αm	4Y + 4H	1.42×10^{14}	2.85×10^{14}
βm	5Y + 1H	1.78×10^{14}	2.14×10^{14}
p1	15Y + 11H	1.81×10^{14}	3.13×10^{14}

b. Residues available for biotinylation:

	Residues per molecule	Total residues per µg
αm	12K + 8N + 7Q + 3R	1.03×10^{15}
βm	14K + 5N + 8Q + 9R	1.24×10^{15}
p1	23K + 36N + 29Q + 15R	1.24×10^{15}

5.1.1 Iodination

0.4 -1µg of HPLC purified IL-1αm (αm) and IL-1βm (βm) were iodinated for 15min using the Iodobead method (Markwell). Mock-digested p1 vlps, Irrelevant protein (discard fractions off the HPLC gradient which contain various yeast proteins and only trace amounts of IL-1) and 0.1% BSA were also iodinated as control preparations. Protein concentrations were estimated by comparison with standards on coomassie and silver stained gels. (as in Ch4). Unincorporated isotope was removed by extensive dialysis into PBS:0.1% azide. If the protein to be labelled was at <500ng/ml, the solution was allowed to sit for at least 2h after removal of the iodobead in order to allow the active labelling

reactants to disappear. 0.1% BSA was then added prior to dialysis in an attempt to minimise loses due to protein adhering to the dialysis membrane. Specific activities obtained were routinely in the range 0.7–1 x 10^6 cpm/pM for ^{125}I-αm, ^{125}I-βm and ^{125}I-p1, similar to IL-1 iodination figures reported in the literature. The specific activity of the irrelevant protein sample was difficult to calculate but was estimated to be in roughly the same range as the other proteins.

5.1.2 Biotinylation

Samples of IL-1βm, 2μg (pre HPLC) and 0.4μg (HPLC purified), were labelled with biotin at a ratio of 10:1 (w/w). Biotinlyated protein in PBA was visualised on electroblotted 15% PAGE gels, using enhanced chemiluminescence. Some remaining p1 in the pre-HPLC preparation appears in the sample. During FACS analysis of receptor binding, addition of unlabelled p1 protein to the incubation buffer should minimise any non-specific binding caused by the biotinylated p1.

Fig. 5.1 shows representative 15% SDS-PAGE gels of unlabelled and labelled proteins.

FIGURE 5.1

Purity of Labelled Proteins as Visualised on 15% SDS-PAGE Gels

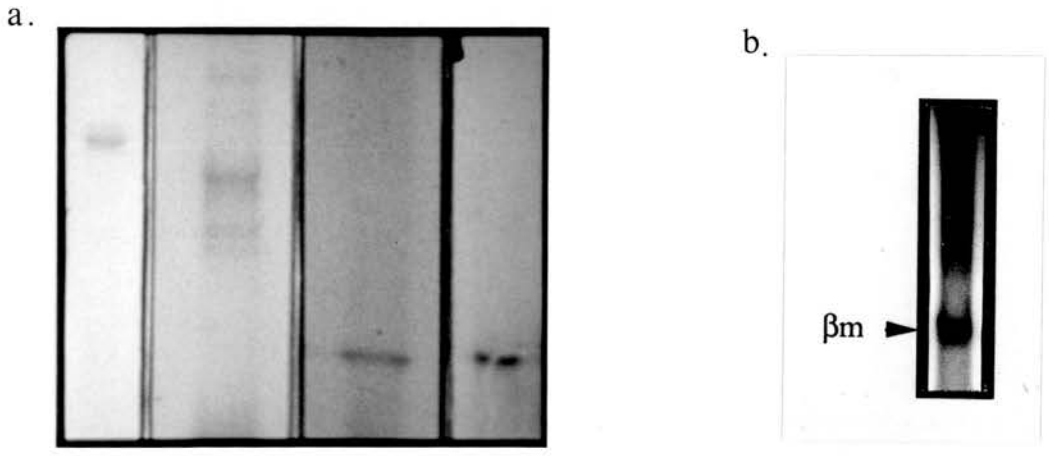


Figure 5.1
(a) ^{125}I-labelled detected by autoradiography of dried gels; p1 (lane 1); irrelevant yeast proteins (Lane 2); IL-1αm (lane 3); IL-1-βm (lane 4);
(b) biotin-labelled IL-1βm detected by enhanced chemiluminescence.

5.2 Bioactivity of labelled rIL-1

All the cells which were available to me for assaying IL-1 activity, possess the type I receptor. The effect of labelling on IL-1 binding to the type II receptor, as found on macrophages, could not therefore be determined by these assays.

Iodination did not significantly reduce the bioactivity of either IL-1α or IL-1β as assayed by cartilage degradation and activity profiles were similar to those shown in Fig. 4.15. Biotinylation did however greatly reduce the bioactivity of IL-1β in the thymocyte assay although mock-biotinylation did not (Fig. 5.2) and it may be that biotin is partially masking the IL-1β binding site.

FIGURE 5.2

Effect of Biotinylation on IL-1β Stimulation of Thymocyte Proliferation

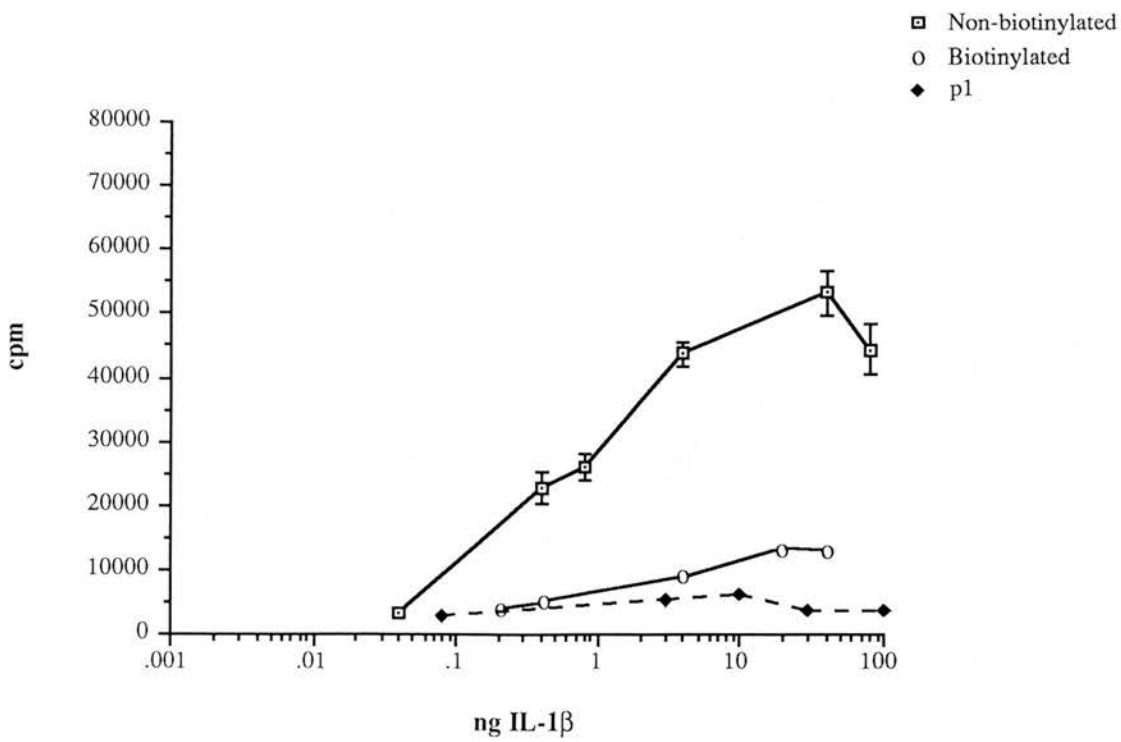


Figure 5.2.
The bioactivity of biotinylated IL-1β was determined in the standard ovine thymocyte proliferation assay.

5.3 Identification of Macrophages, Dendritic Cells and Lymphocytes within Afferent Lymph Cell Populations

Afferent lymph may contain some blood or tissue derived macrophages. It is therefore important to be able to establish that any IL-1 binding detected is not due to the presence of contaminating macrophages. Although there is no absolute method by which to distinguish macrophages and dendritic cells, cell surface markers and cytochemical staining can be used as indicators.

5.3.1 Cell Collections

5.3.1.1 Macrophages

Ovine alveolar macrophages were obtained either under anaesthetic by lung washout with 200ml HBSS at the time of cannulation, when possible, or from unrelated sheep by bronchoalveolar lavage with 2 x 500ml HBSS at post mortem. Cells were purified by Lymphoprep cushioning and resuspended in PBA.

5.3.1.2 Afferent Lymph Dendritic Cells and Lymphocytes

Pseudoafferent lymph from cannulated prefemoral ducts was collected into sterile, heparinised bottles. Polymorphonuclear leucocyte and red blood cell counts had returned to minimal levels by 3-6 days post cannulation and lymph was normally not utilised prior to this. On average, 24h lymph collections produced 80-150ml lymph containing 2 x 10^6 cells/ml of which 5-10% were DC. Centrifugation over a discontinuous (14.5% over 20% w/v) metrizamide gradient in Iscoves serum free medium enriched the DC fraction. The upper interface population consisted of >80% DC, the remainder being lymphocytes. The lower interface consisted of lymphocytes and some dendritic cells. Washed DC and lymphocytes were resuspended in PBA;0.1% Na azide.

5.3.2 Analysis of Surface Phenotype by Immunofluorescence

A number of anti-ovine monoclonal antibodies were available in the department, some of which proved useful for differentiating between Mϕ and afferent lymph DC by FACS analysis. Table 5.2 shows the results of FACS analysis of 10^5 cells from gated populations, the gates used being shown in Fig. 5.3.A. Fluorescence is graded from undetectable (–) or faintly positive (+), to strongly positive (++++). Normal mouse serum and an antiviral antiserum, 1D10, which does not bind to Mϕ, were used as negative controls. Some antisera produced both

negatively and positively reacting populations, in which case each population is graded separately (eg. +/–). The percentage of positive cells in each case is indicated in parentheses. Fig. 5.3.B shows examples of FACS profiles obtained with VPM5 and VPM32, the antisera which can best be used to distinguish between Mϕ and DC. Analyses carried out on total and gated populations show minimal macrophage contamination of DC preparations. Lymphocyte contamination in different preparations ranged from 15 - 20%.

TABLE 5.2

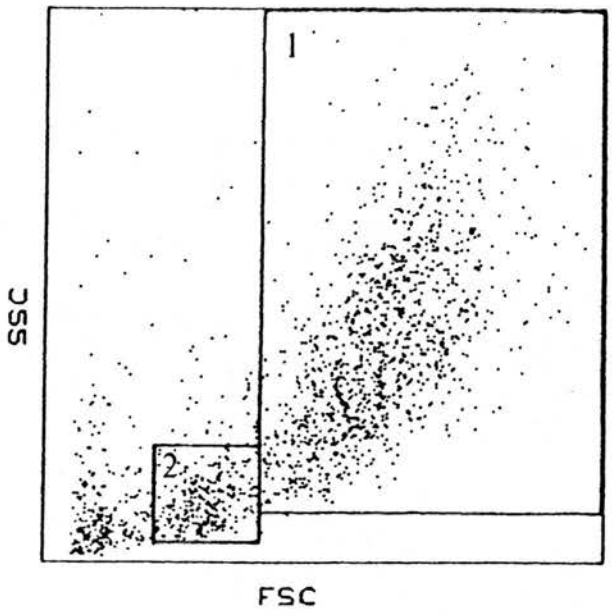
Determination of Surface Phenotype of Alveolar Macrophage and Afferent Lymph Cell Populations by Immunofluorescence

Ab	Ag	Isotype	Macrophage	Dendritic	Lymphocyte
CC14	CD1	IgG_1	++	++++	++++
VPM5	CD1	IgM	–	+++	–
SBUT4	CD4	IgG_{2a}	++	–	+++
SBUT8	CD8	IgG_{2a}	+/–(<10% +ve)	–	+++
VPM13	μ-chain	IgM	–	+/–(<10% +ve)	++
VPM32	CD14(?)	IgG_{2a}	+++	+	–
VPM65	CD14(?)	IgG_1	+++	+	–
VPM54	DRα	IgG_1 Class II	++	++++	–
1D10	EV1 p25	IgG_1	–	–	–
NMS	–	–	–	–	–

Table 5.2
Surface phenotype was determined by FACScan analysis. Isolated cell populations were incubated either with FITC conjugated primary antibody or non-biotinylated antibodies which were detected with FITC-conjugated $F(ab)_2$ fragment of rabbit anti-mouse immunoglobulin. Washed cells were analysed by flow cytometry of 10^4 cells within gated populations). VPM32 and VPM65 display the characteristics expected of anti-CD14 monoclonal antibodies but N-terminal analysis is still required for confirmation.

FIGURE 5.3

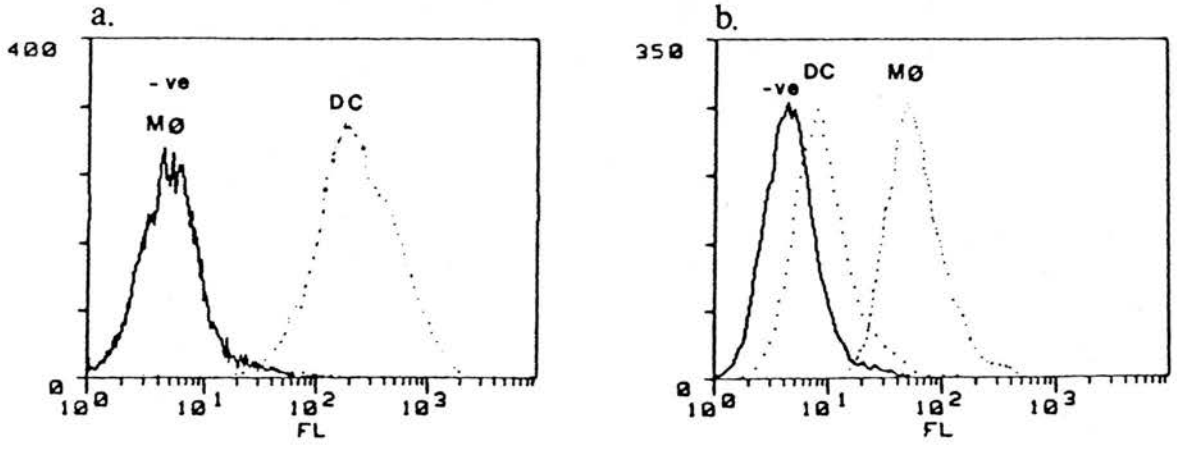
A. Gated Cell Populations for Immunofluorescence Analysis by FACS



Cell populations were isolated on the basis of their forward scatter (FSC) and side scatter (SSC) profiles when analysed by FACScan using the Consort 30 Data management system. The gate settings shown were used for all analyses of individual cell populations.

1. Alveolar macrophages (Mϕ) and Dendritic cell (DC) gate
2. Lymphocyte (L) gates

B. FACScan Profiles of Macrophages and Afferent Lymph Dendritic Cells Reacted with VPM5 and VPM32



Positive fluorescence is indicated by a shift to the right on the FSC axis.

a. Positive fluorescence of DC and negative fluorescence of macrophages reacted with VPM5;

b. Positive fluorescence of macrophages and minimal fluorescence of DC reacted with VPM32.

5.3.3 Distinguishing Mϕ from DC by Cytochemical Staining

5.3.3.1 Leishman's Stain

Cytospins stained with Leishman's stain show the comparative distribution of the various cell populations in unfractionated afferent lymph and whole blood (Fig. 5.4.a).

5.3.3.2 Giemsa Stain

Giemsa staining shows DC to be lightly staining, veiled cells or cells with pseudopodia (Fig.5.4.b). Many of the nuclei appear kidney shaped. The stain is not very useful for DC/Mϕ differentiation purposes but is useful for counterstaining cells which have been exposed with radiographic emulsion (see Sect. 5.4)

5.3.3.3 Non-specific Esterase Stain (NSE)

Mϕ stain very darkly for non-specific esterase whereas DC stain much less strongly, as seen in Figs. 5.5. Whereas Mϕ stain fairly uniformly, a number of different patterns of DC staining were seen, the most obvious being indicated as follows:

Large cells showing;

(a) dark cytoplasmic staining of various distributions
(b) light cytoplasmic staining
(c) extremely faintly staining veiled cells with no visible granules

Vacuoles can be seen in some cells and all of the above, apart from type (e), can display dendritic processes as can be clearly seen in Fig. 5.5.B

Smaller cells with dendritic morphology showing;

(d) mainly intermediate reticular type staining.

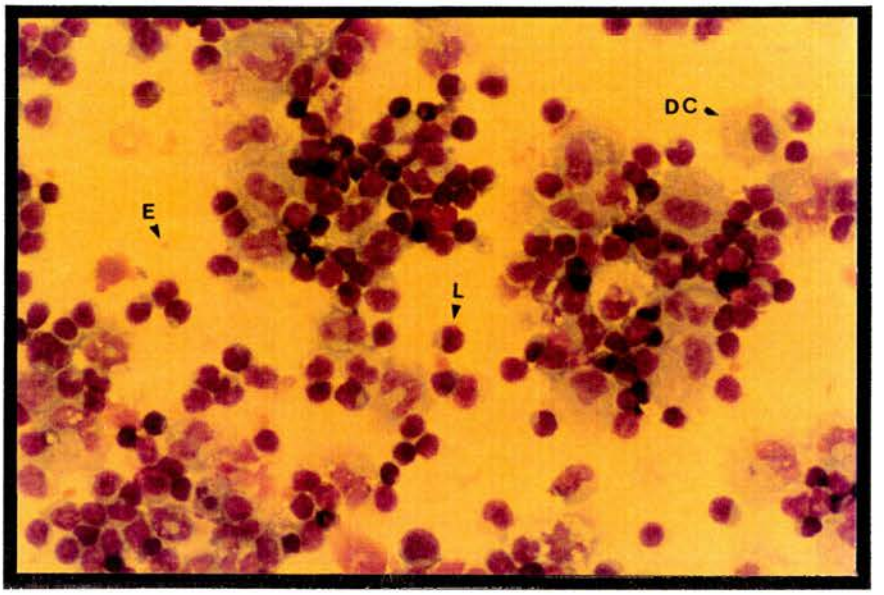
Lymphocytes all stain pale green and are esterase negative.

NSE is the most useful of the cytochemical stains for differentiating between Mϕ and DC.

FIGURE 5.4

Unfractionated Afferent Lymph and Top Fraction Metrizamide Cell Populations

a.



b.

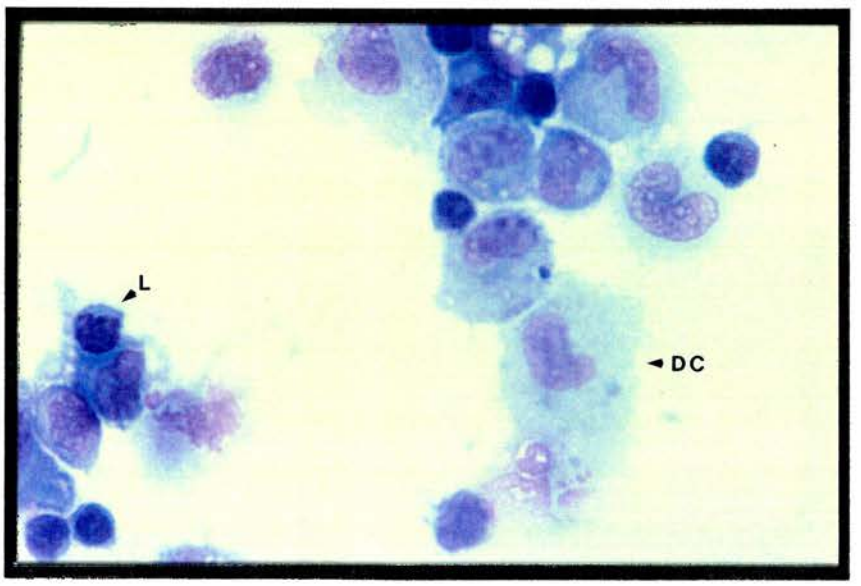


Figure 5.4
Cells were stained by standard staining techniques detailed in Chapter 2.11.4

a. Leismans' stained preparation of unfractionated lymph. DC - dendritic cell; L - lymphocyte; E - erythrocyte. (x 320)

b. Giemsa stained preparation of isolated afferent lymph dendritic cells. (x 780)

FIGURE 5.5

Staining Macrophages and Dendritic Cells for Non-specific Esterases

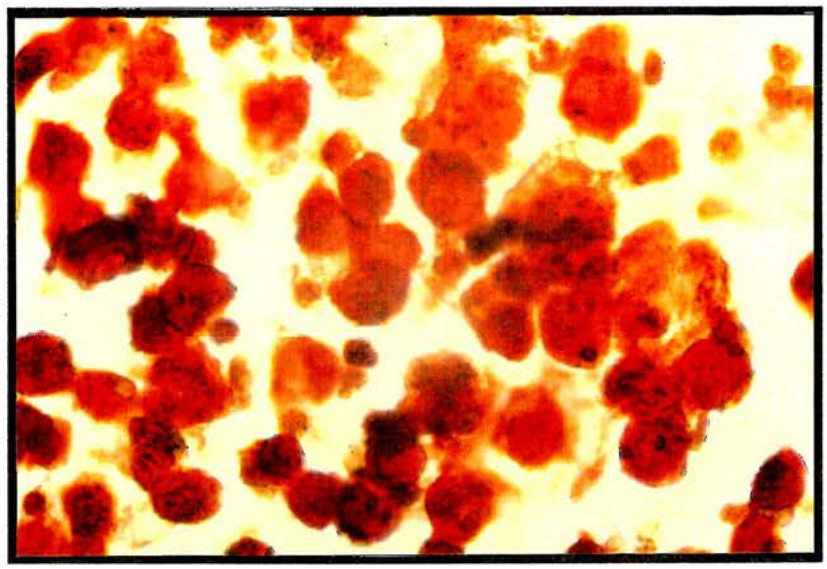
Figure 5.5

Cytospun cells were stained for non-specific esterase and counterstained with methyl green as per Chapter 2.11.4.5. Cells were viewed under a light microscope at x500 or x780 magnifications.

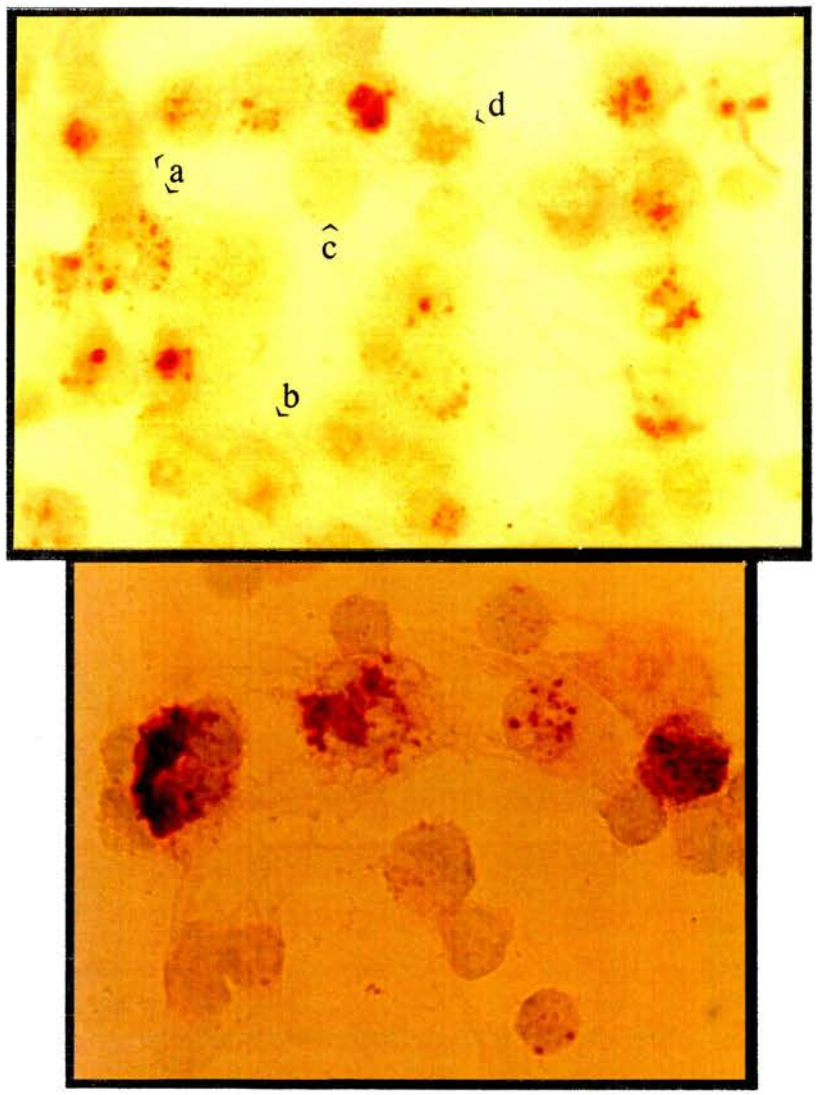
A. Alveolar macrophages stain dark red and are strongly positive (x 500).
B. Various dendritic cell staining patterns, indicated as a - d (x 500), are detailed in the main text, Sect 5.3.3.3. Cells at x 780 magnification show dendritic processes.

Lymphocytes stain pale green.

A.



B.



5.3.3.2 Acetylcholinesterase Stain (AChe)

Within the sheep epidermis, AChe activity is detected specifically in Langerhans' cells (Hollis and Lyne, 1972) and it is the cell membrane and not the cytoplasm which stains positively for AChe. Macrophages and lymphocytes do not contain AChe and only take up the counterstain, methylene blue. This stain is of use for positive identification of Langerhans' or Langerhans'-derived cells but it can be seen from Fig. 5.6 that only a very small proportion (~2%) of isolated afferent lymph DC show positive staining for AChe. The number of non-staining DC means that AChe content can not be used to distinguish between macrophages and DC within these populations.

Three apparently distinct patterns of AChe staining are seen;

(a) large cells with rounded or kidney-shaped nuclei and short dendritic processes, which show overall strong staining
(b) cells similar to the above but which show more diffuse and fainter staining
(c) cells, many with irregularly shaped nuclei, which show granular staining which may be either round the circumference of the cell or in the form of distinct punctate staining. The latter sometimes appears to be associated with longer processes.

These staining patterns are highlighted on counterstained samples in Fig 5.6.A.

Isolated DC which have been stained for AChe, but not yet counterstained, show the presence of long dendritic processes on many of the cells when viewed under phase contrast conditions (Fig. 5.6.B). It is extremely difficult to distinguish these processes after counterstaining. Only very few of these latter cells show positive AChe staining and those that do, seem to exhibit the type (c) staining pattern detailed above.

FIGURE 5.6

Staining Afferent Lymph Dendritic Cells for Acetylcholinesterase

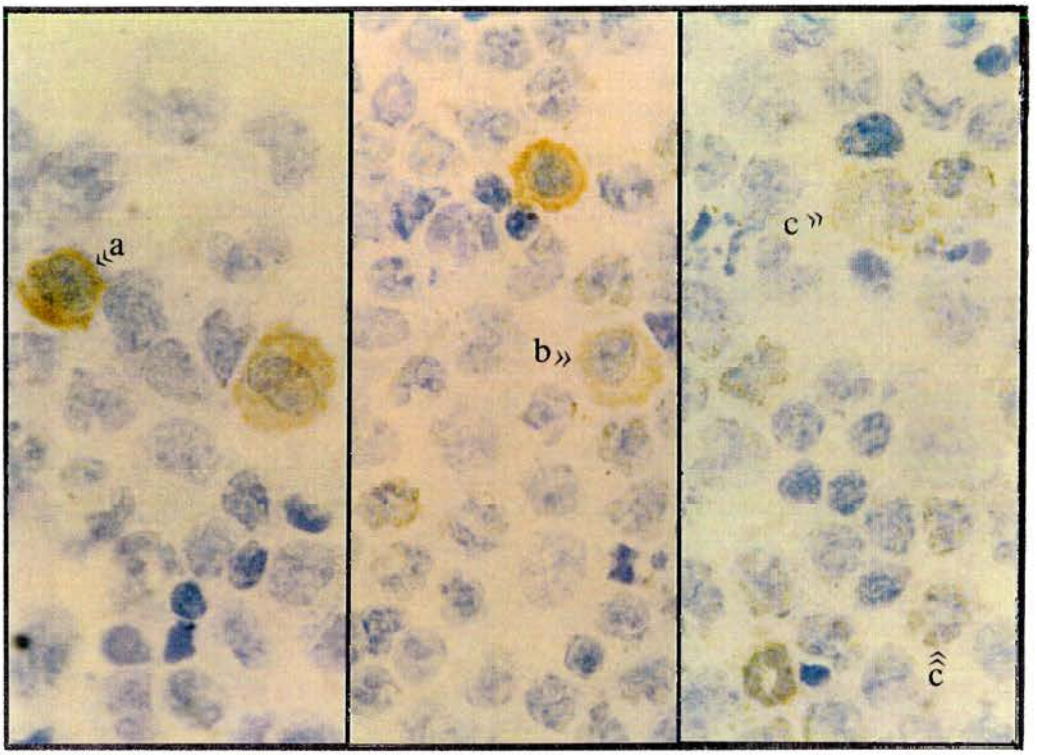
Figure 5.6

Cytospins of isolated afferent lymph DC populations were stained for AChe as per Sect. 2.11.4.4, and viewed under a light microscope. Magnifications at which photographs were taken are given.

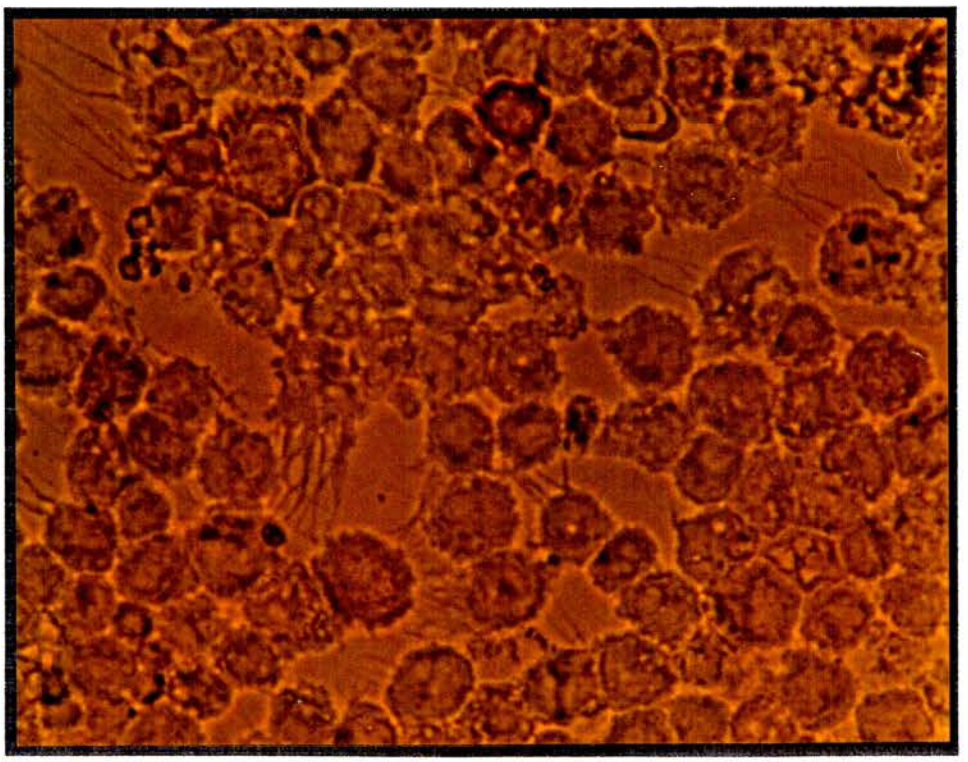
A. DC stained for AChe and counterstained with Methylene blue. Staining patterns a - c, as described in Sect. 5.3.3.4 in the text, are indicated (x 320).

B. DC stained for AChe, but not counterstained, viewed under phase contrast conditions. AChe positive cells appear dark ochre (x 500).

A.



B.



5.4 Detection of IL-1R on Alveolar Macrophages

Alveolar macrophages are known to possess IL-1 receptors and as such are a good positive control for the detection of specific binding by the labelled proteins. Both biotinylated and iodinated IL-1 preparations were used for detecting IL-1R on Mϕ. Binding of the biotinylated protein was detected by FACS analysis. Bound ^{125}I-IL-1 was detected either on cytospins as above or by counting the radioactive samples.

5.4.1 Detection with Fluorescent Ligand

1 x 10^5 Mϕ in PBA were incubated with up to 100ng biotinylated IL-1β and the bound ligand detected with phycoerythrin-conjugated streptavidin. Fluorescence analysis of the gated macrophage population showed minimal positive signal even at the highest IL-1β concentration (Fig. 5.7). The biotinylation method used had been adapted from the standard method for antibody labelling (Pierce Ltd.). Molar ratios of the reactants were maintained and the protein labelled successfully (see Sect. 5.1.2). However, in view of the comparatively minute amounts of protein being labelled here (≤1μg IL-1 vs. mg amounts of antibody), there was a possibility that these conditions might not be ideal for maintaining acitivty. Three different preparations were made with titrated amounts of biotin (10 x more; 10 x less and 100 x less) were all similar to the initial preparation and none gave a stronger fluorescence signal. These results corroborate the low biological activity found for biotinylated IL-1β.

FIGURE 5.7

Detection of IL-1R on Alveolar Macrophages using Biotinylated IL-1β

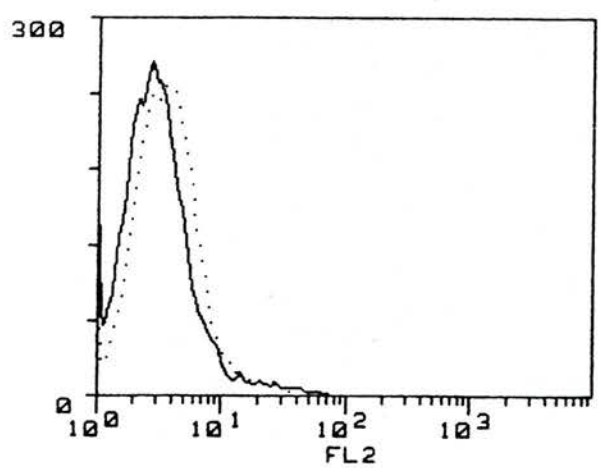


Figure 5.7

1 x 10^5 macrophages were incubated with 100ng biotinylated IL-1β in PBA. Bound ligand was deteced with Streptavidin-PE and the macrophage gated population analysed by FACScan.

5.4.2 Radiographic Detection

In order to establish that the ^{125}I-IL-1 would bind to its receptor, 2 x 10^6 Mϕ which had been incubated for 2h at 4°C with 5ng ^{125}I-IL-1 in PBA were washed with cold PBA and cytospun at 500rpm for 5min. Bound ^{125}I-IL-1 was detected by exposing the slides with Amersham LM-1 emulsion. Sodium azide was included in all solutions to prevent internalisation of bound ligand. Developed cytospins were counterstained with Giemsa.

Representative pictures of iodinated protein binding to Mϕ are seen in Fig. 5.8. No bound radioactivity was detectable on cells which had been incubated with any of the control preparations (^{125}I-p1 shown). ^{125}I-αm binding is detectable on 14% of cells and ^{125}I-βm on 24% of cells. On an individual cell basis, the amount of material bound varies greatly but IL-1α binding (Fig. 5.8.a), at ≤200 grains per cell, appears to be less concentrated than IL-1β (Fig. 5.8.b), at up to >500 grains per cell. Specific competition with ≥1μg unlabelled material greatly reduced ^{125}I-IL-1 binding to Mϕ. In addition, IL-1β competed with ^{125}I-αm binding but IL-1α was very inefficient at competing with ^{125}I-βm binding. The ^{125}I-IL-1 binding was not inhibited by the presence of unlabelled p1.

Having been able to detect specific IL-1 receptors on macrophages, the iodinated reagents could now be used for establishing whether afferent lymph dendritic cells express IL-1R.

5.5 Detection of IL-1R on Afferent Lymph Dendritic Cells

Iodinated IL-1 preparations were used for detecting IL-1R on afferent lymph DC. It had been hoped that biotinylated protein could be used for fluorescence analysis but in view of the lack of positive signal on macrophages this was not however feasible. Bound ^{125}I-IL-1 was detected either on cytospins as above or by counting the radioactive samples.

5.5.1 Cytospins

Incubation of DC with ^{125}I-αm or ^{125}I-βm as above revealed a low level of binding to the cells (Fig. 5.9). Cytospins show ≤0.5% of DC with grains and ^{125}I-αm and ^{125}I-βm binding patterns appear to be slightly different. More cells were ^{125}I-αm positive but with fewer grains per individual cell; ≤80 on average. Positive ^{125}I-βm cells had 94 ± 43 grains, divisible into two groups; <90 grains (54.2 ± 14.6), and ≥90 grains (130.9 ± 29), over individual cells. None of the positive cells appear to display the classical veiled dendritic morphology and they are mainly faintly staining, 'non-granular' cells. There is little evidence of macrophage contamination. Photographs of dendritic cells to which ^{125}I-IL1 has bound are shown in Fig. 5.16 on pg 200.

FIGURE 5.8

Binding of ^{125}I-Labelled Proteins to Alveolar Macrophages

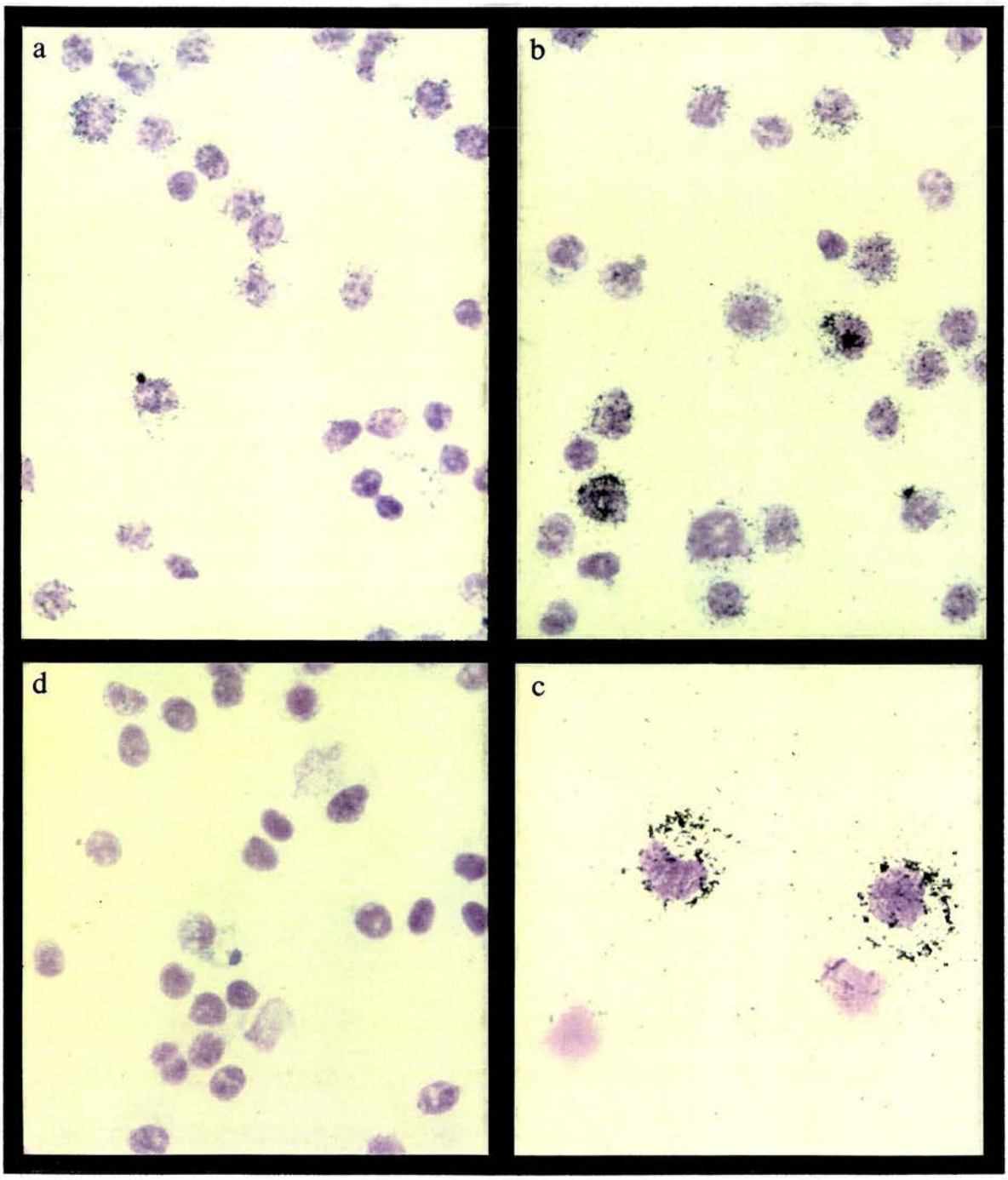


Figure 5.8
Isolated alveolar macrophages were incubated with 5ng ^{125}I-IL-1 for 2h at 4°C and unbound material removed by washing with cold PBA. Cells were cytospun and dipped in LM-1 emulsion. Slides were developed after 4 weeks exposure and counterstained with Giemsa. Magnifications are those at which the photographs were taken

a. IL-1α (x500); **b.** IL-1β (x500); **c.** IL-1β (x780); **d.** p1 (x500).

5.6 Establishing an Assay for the Binding of ^{125}I-IL-1 to Mϕ and DC

A reproducible assay procedure for quantitation of bound ^{125}I-IL-1 had to be established, from which the number of binding sites per cell and the binding affinity could be calculated (Scatchard). Cytospins would further define the binding on an individual cell basis.

5.6.1 Binding Assay

All binding experiments were done in the presence of 0.1% Na azide to prevent internalisation of the bound ligand. Initial experiments involved incubation of Mϕ, DC or lymphocytes with ^{125}I-IL-1. Unbound radioactivity was removed by sequential washing of the samples with ice cold PBA in either 1.5ml Eppendorf tubes or FACS tubes. Half of the sample was counted and the rest cytospun and exposed with LM-1 emulsion. It became apparent that unbound ^{125}I-IL-1 was adhereing to the plastic tubes hence giving falsely high cpm figures. Unbound material was subsequently removed by centrifugation through an oil gradient (see below).

For Scatchard analysis all incubations were carried out in final volumes of 100µl in 1.8ml screw cap Eppendorf tubes using a rotating mixer for 2.5h at 4°C. Fig. 5.9 shows that binding has reached equilibrium by this time. Cells to be counted and those to be cytospun were incubated independently, the former with 0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 50ng ^{125}I-Protein and the latter with 5ng ^{125}I-Protein. Cells for counting were washed three times with 1.8ml ice cold PBA, resuspended in 100µl PBA and spun through 300µl phthalate oil (dibutyl phthalate:bis(2-ethylhexyl) phthalate 1.5:1 v/v), in Sarstedt 0.3ml microsedimentation tubes, to remove the remaining unbound labelled protein, modifying the method of (Bomsztyk et al., 1989b) slightly. The tube was frozen in a dry ice/ethanol bath and the tip containing the cell pellet cut off and counted. Tests with varying concentrations of labelled protein established that provided the oil was frozen prior to loading the sample, microfuging for 110sec at 13500rpm would pellet the cells efficiently without contamination by unbound material. (Surface tension effects cause warm oil to enclose the sample as a bubble and unbound radioactivity is hence drawn through the oil while the phases separate during spinning.)

FIGURE 5.9

Determining the Time Required to Establish Binding Equilibrium

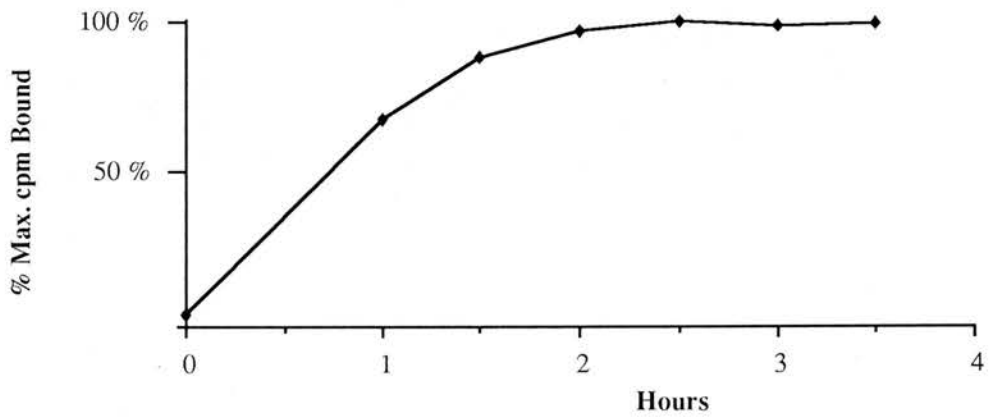


Figure 5.9
Samples containing 2 x 10^6 dendritic cells were incubated at 4°C for 2.5h with 5ng ^{125}I-IL-1β. Bound radioactivity was determined at intervals, as shown. 100% binding represents 1500cpm.

Binding of IL-1α to DC and of IL-1α and IL-1β to Mϕ all reached equilibrium in less than 2h.

5.6.2 Cytospin analysis

Cells for cytospinning could not be centrifuged through oil and were given two additional washes with PBA prior to spinning. Most of the remaining free labelled protein could be washed off the slide. Residual unbound radioactivity would be visualised as background. Cytospins were fixed with cold acetone and dipped in Amersham LM-1 emulsion diluted 1:2 with water. Grains seen after short term exposure were very small and slides were left for 4-8 weeks, depending on the expected signal, before developing.

Staining of cytospins: Non-specific esterase staining was done prior to dipping the slides and Giemsa staining after developing the exposed slides.

5.7 Quantitation of IL-1 Receptors on Alveolar Macrophages and Afferent Lymph Dendritic Cells and Lymphocytes

5.7.1 Specificity of Binding

Cells were incubated with ^{125}I-IL-1 in the presence or absence of an excess of cold competitor. Representative binding curves, of ^{125}I-IL-1β binding to Mϕ (Fig. 5.10), demonstrate the specificity of binding, the presence of the unlabelled material dramatically reducing the binding of ^{125}I-IL-1. Binding of ^{125}IL-1α and ^{125}IL-1β to Mϕ and DC could all be specifically inhibited by cold competition.

FIGURE 5.10

Demonstration of the Specificity of ^{125}I-IL-1 Binding

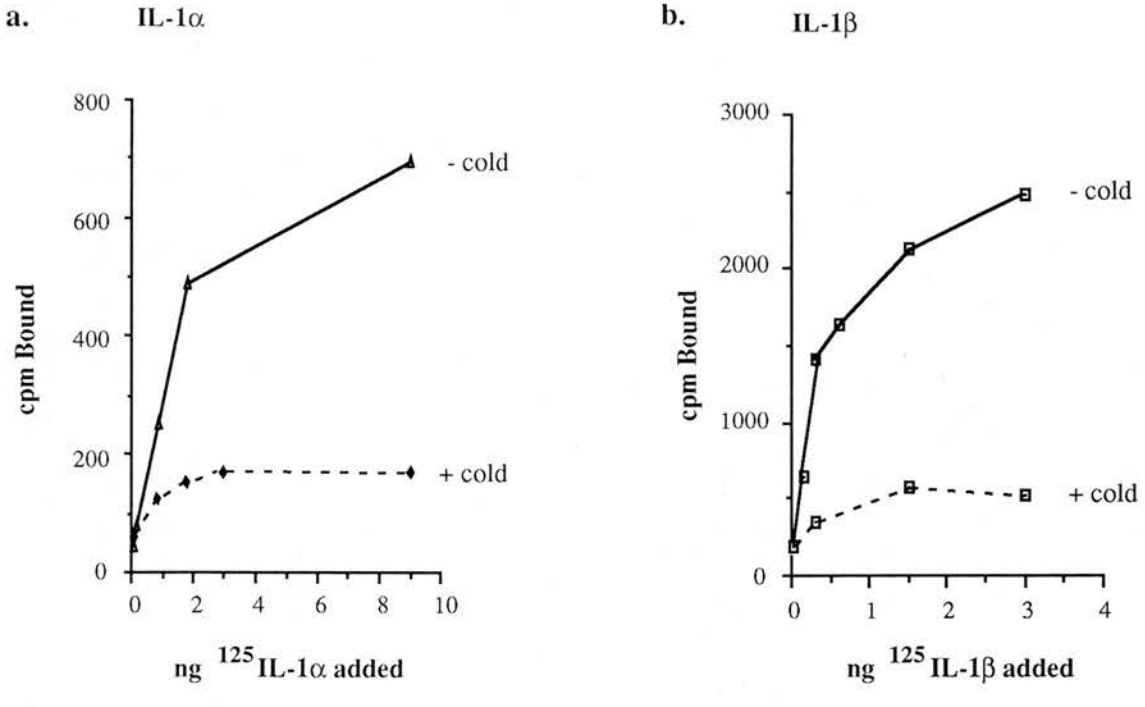


Figure 5.10
Macrophages were incubated with increasing concentrations of ^{125}I-IL-1 in the presence or absence of 250ng unlabelled IL-1.

a. IL-1α; **b.** IL-1β

5.7.3 Scatchard Analysis of IL-1 Receptor Binding

Both Mϕ and DC Scatchard plots are biphasic curves, indicating the presence of high and low affinity IL-1 binding sites. Far more IL-1 sites are detected on Mϕ than on DC. Mϕ appear to have a significantly greater affinity for ^{125}I-βm than ^{125}I-IL-1αm and bind 5 - 6 times more ^{125}I-βm. Resting DC, on the other hand, bind ^{125}I-αm and ^{125}I-βm to a similar degree. ^{125}I-p1, ^{125}I-Irrelevant-protein and ^{125}I-FXa do bind to Mϕ and DC, but non-specifically and only at high concentrations (>500ng/ml). Afferent lymphocytes, at 2 x 10^6 cells per incubation, showed some binding of ^{125}I-αm and minimal binding of ^{125}I-βm. Representative Scatchard plots of ^{125}I-IL-1 binding are shown in Fig. 5.11 and Table 5.3 summarises the individual cell and Scatchard analysis data obtained.

TABLE 5.3

Quantitation of IL-1 Binding Sites on Ovine Alveolar Macrophages and Afferent Lymph Dendritic Cells and Lymphocytes

	Cell	Grains/cell	% +ve	Sites/cell	K_d pM
IL-1αm:					
	Mϕ	30 - 200	14	2600 ± 98	56 ± 11
	DC	41 ± 25	<0.5	510 ± 36	30 ± 4
	L	50 ± 30	<1.0	417 ± 147	181 ± 61
IL-1βm:					
	Mϕ	30 - >>500	24	16500 ± 707	4.6 ± 1
	DC	94 ± 43	<0.5	350 ± 145	160 ± 58
	L	<<<	<<<	≤50	not calc.

Table 5.3
Grains/cell indicates the number of grains detected over individual cells for incubation of 5ng ^{125}I-IL-1 with 2 x 10^5 cells. Sites/cell and K_d were determined by Scatchard analysis (see Fig. 5.11 for representative plots).

FIGURE 5.11

Representative Scatchard Plots of ^{125}I-IL-1 Binding to Alveolar Macrophages and Resting Afferent Lymph Dendritic Cells

Figure 5.11

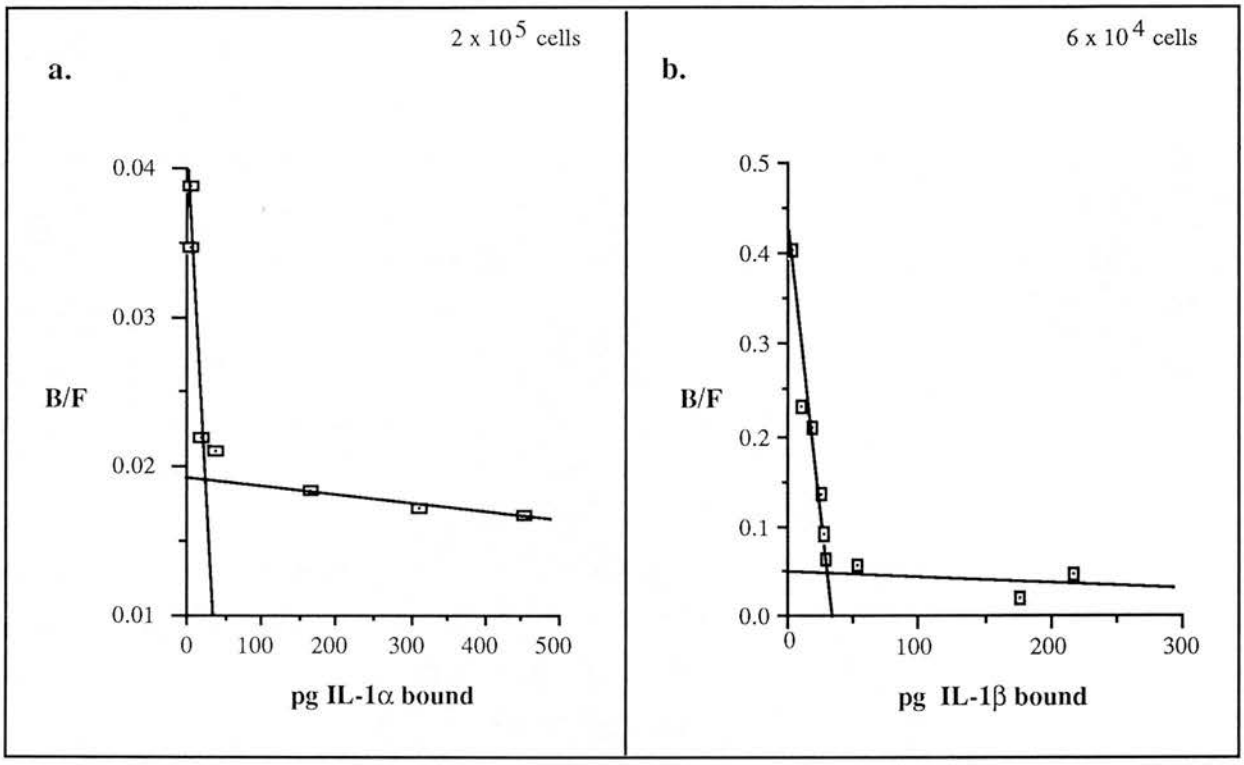
Cells were incubated with ^{125}I-IL-1 and bound radioactivity detected as described in Sect. 5.7.1. Scatchard analyses were carried out as per Scatchard (1949).

a. ^{125}IL-1α binding to macrophages; **b.** ^{125}IL-1β binding to macrophages

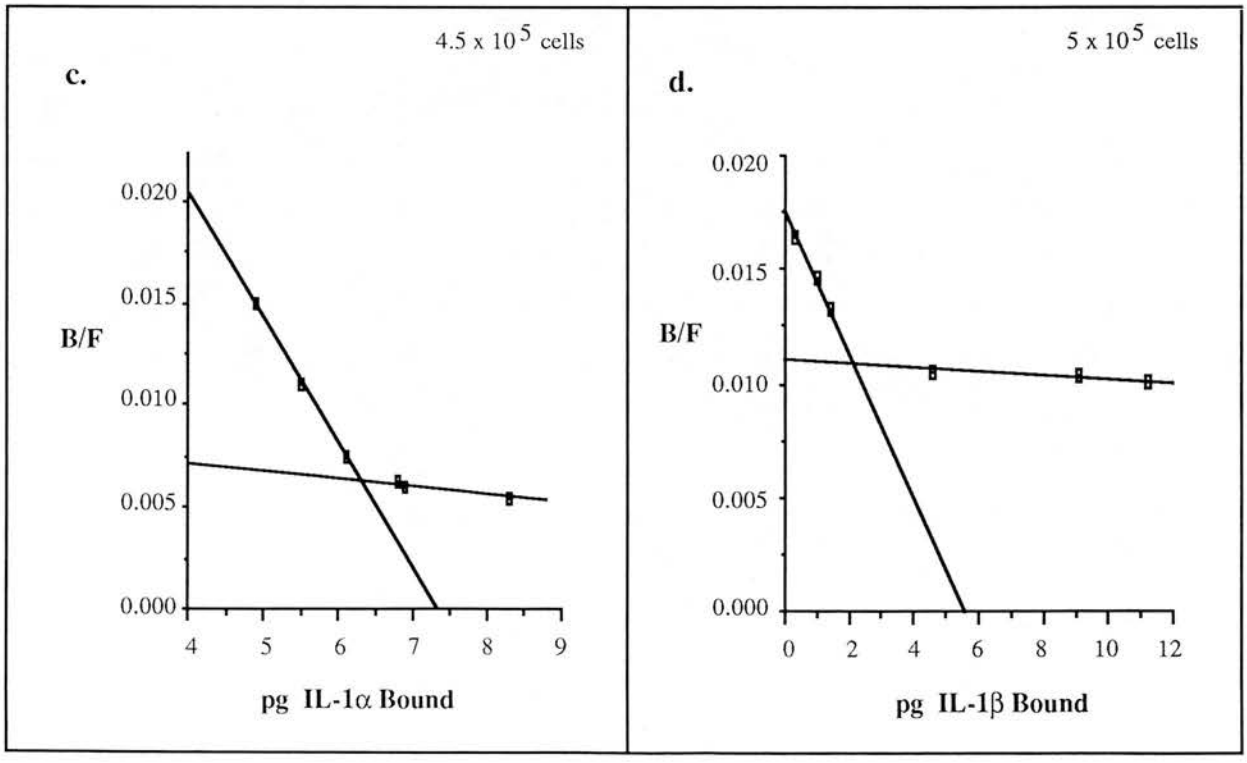
c. ^{125}IL-1α binding to dendritic cells; **d.** ^{125}IL-1β binding to dendritic cells

FIGURE 5.11

Macrophages



Dendritic Cells



5.7.4 Limits of detection

Calculations based on the specific activity of labelled IL-1 suggest that the lowest numbers of receptors detectable by Scatchard analysis would be 150sites/cell and 50sites/cell for incubations with 2 x 10^5 cells and 2 x 10^6 cells respectively.

5.7.5 Reproducibility of the Assay

Due to the small numbers of DC obtainable, especially over short time periods if the lymph flow rate was low, it frequently proved difficult to have enough cells for duplicate or triplicate incubations. It was imperative, therefore, to establish that standard deviations obtained with this binding assay were low. Quintuplicate samples of 2 x 10^5 DC were incubated with varying concentrations of ^{125}I-IL-1. Because of the number of cells involved, the assays had to be carried out with different DC collections. Standard errors were of the order of 9% and judged to be acceptable.

5.7.2 Dissociation Kinetics of IL-1 Binding to Dendritic Cells

Full dissociation kinetics were not calculated but DC samples which had been washed were split and half spun through oil immediately. The other half was incubated overnight at 4°C prior to separating the unbound material. About 9% bound IL-1α and 25% bound IL-1β had dissociated, indicating a higher affinity of the receptors for IL-1α.

5.8 Effect of Ovalbumin Challenge on IL-1R Expression by Afferent Lymph Dendritic Cells and Lymphocytes

5.8.1 Resting State Prior to Secondary Challenge

6 sheep were boosted with 50μg ovalbumin and the pseudoafferent ducts cannulated 6 weeks later. Cell output and IL-1 binding was monitored over 2-3 weeks. Total cell output showed little variation and averaged 1.9(±0.08) x 10^6 cells/ml over this period. The binding assay results were similar to those shown in Fig. 5.11 and were included in the data in Table 3.1 above. Although there was some variation between animals with respect to ^{125}I-βm binding, sequential samples from any one animal showed no significant variation. Variations in ^{125}I-αm binding were less.

5.8.2 Responsiveness of Sheep to Ovalbumin Challenge

Blood monocytes were separated from jugular vein samples. Their ability to proliferate when incubated with ovalbumin was measured by incorporation of ^{3}H-Thymidine. A typical response curve is shown in Fig. 5.12. All sheep used were found to be adequate responders to ovalbumin challenge.

FIGURE 5.12

***in vitro* Proliferative Response to Ovalbumin by Mononuclear Cells from Ovalbumin Primed Sheep**

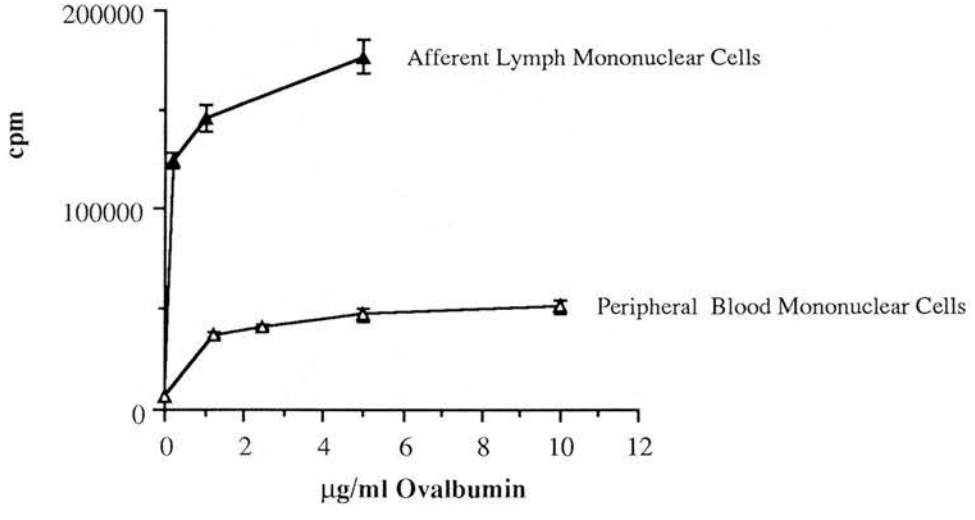


Figure 5.12
Peripheral blood mononuclear cells and unfractionated afferent lymph were incubated with ovalbumin for 5d. Cultures were pulsed with 1µCi ^{3}H-T over the final 7h. Incorporated radioactivity on harvested cells was measured by scintillation counting. The proliferative response of PBMC to ConA, used as a positive control, was typically ten-fold greater than the ovalbumin response.

5.8.3 Secondary Ovalbumin Challenge

3 sheep were injected intradermally with 50μg ovalbumin in PBS at sites round the cannula. Afferent lymph was to have been collected at four hourly intervals for the first 24h and thereafter at 24h intervals. In practice, one cannulation, (SH1), stopped flowing by 6h and a second, (SH2), by 25h. From the information gained from these two sheep, collections from the third sheep were taken at 4h and 18h and then at 24h intervals for 10 days (SH3-1), by which time total cell counts and IL-1 receptor expression had returned to resting levels. This sheep was re-challenged with ovalbumin on day 10 (SH3-2), and lymph collected at 24, 48 and 72h. A couple of episodes of lymph clotting within the cannula occurred during this repeat challenge, after each of which the canula was manually cleared and heparin administered. Lymph collection was only restarted after excess heparin had been allowed to flow out of the tubing. Heparin concentrations thus remained unaltered throughout collections. It will be seen from the results that the single heparin injection did not induce detectable IL-1R. The cannula blocked irreversibly overnight between 58h and 72h after challenge. As a result of these clotting episodes, only small volumes of lymph were obtained from SH3-2.

5.8.3.1 Cell Output in Afferent Lymph

Resting state cell output averaged 1.9 x 10^6 cells/ml. In response to secondary challenge, total cell numbers in the three sheep studied had risen by 4h, (only slightly in two sheep but nearly two-fold in one, SH2), followed by a fall to less than baseline by 8h and a return to normal by 12h. Only one animal, SH3, could be followed for longer than 24h. From 18h the SH3-1 cell numbers again increased to reach a maximum of 4 times baseline by 66h, after which the counts decreased slowly and had returned to normal by day 8. During the repeat ovalbumin response, the SH3-2 cell count reached a maximum by 48h. SH2, which had a nearly two-fold increase in total cells by 4h also showed a two-fold increase in the proportion of DC in this sample. Otherwise the only other distinct variation in %DC was in SH3-2 at 24h and 48h. These low numbers may be a result of clots forming within the lymph. Table 5.4 details the afferent lymph cell output. The proportion of AChe positive cells (~0.05%) showed no obvious increase at any point during the cannulation.

The resting rate of DC influx into the node if flow is good is 5 x 10^5 DC/h. This increases to >1.3 x 10^6 DC/h over the first 4h and >3.8 x 10^6 DC/h between 24h and 72h after secondary challenge.

TABLE 5.4

Cell Output in Afferent Lymph after Localised Secondary Ovalbumin Challenge

Hours post ova	SH1 ml/h	SH1 Total x 10^6/ml	SH1 %DC		SH2 ml/h	SH2 Total x 10^6/ml	SH2 %DC		SH3 ml/h	SH3 Total x 10^6/ml	SH3 %DC	SH3 DC x 10^4/ml	SH3 AChe +ve/ml
First 2° challenge:													
1 - 0	3.2	1.9	4	#1	1.3	2.0	4		7.1	1.8	4	7.2	90
4	1.3	2.4	6		5.8	3.6	8		10.0	2.1	6	12.6	105
8					3.8	1.7	4						
12					5.3	2.0	4						
18									10.4	1.9	5	9.5	95
24					1.6	2.2	5						
43									10.8	4.0	5	20.0	200
66								*	10.6	7.2	5	36.0	360
90									10.4	5.2	5.5	28.6	260
114									7.5	4.4	5	22.0	220
138									10.0	2.8	5	14.0	140
162									6.5	2.5	5.5	13.8	125
186									6.2	2.0	6	12.0	100
Repeat 2° challenge:													
2 - 0									6.2	1.3	5	6.5	nd
24								#1	2.8	0.9	2.5	2.2	nd
48								#2	1.7	6.4	3	19.2	nd
72									1.3	5.0	4	20.0	nd

Table 5.4
The ovalbumin primed sheep was challenged with 50µg ovalbumin injected locally round the cannulation site.Psuedoafferent lymph was collected into sterile 250ml plastic bottles containing 2.5 x 10^3 units of heparin and 2.5 x 10^4 units each of penicillin and streptomycin. The dendritic cell fraction was isolated by centrifugation onto a 14.5% metrizamide cushion, washed and resuspended in PBA for counting. The percentage of AChe positive cells was established from stained cytospins of isolated DC.

2° = secondary;
#1 = heparinisation of cannula;
#2 = heparin injection.
* = sample clotted on metrizamide;
nd = not determined because of limited cell numbers

5.8.3.2 Binding Assays and Cytospins

When there were enough cells to do so, 2 x 10^5 DC and 2 x 10^6 lymphocytes were incubated for Scatchard analysis with ^{125}I-Protein covering the range 0.05 - 50ng. Otherwise, DC for counting were incubated with 1, 2, 5 and 10ng ^{125}I-Protein and DC for cytospinning were incubated with 10ng ^{125}I-Protein. After secondary challenge, 4, 8 and 12h lymph collections contained very few DC and were only incubated for cytospins. To minimise any bias, incubation tubes were always washed and spun in random order.

5.8.3.2.1 Binding Assays

Throughout the cannulation IL-1β binding to lymphocytes was virtually undetectable. The ~420 IL-1α binding sites detected in resting animals decreased to fewer than 200 throughout the first 4 days of secondary challenge. The small number of contaminating lymphocytes in the DC enriched fraction ($\leq$ 20%) did not therefore contribute significantly to any bound counts detected in the DC population. In addition, non-specific esterase and acetylcholinesterase staining excluded the possibility that this binding detected in this population was due to contaminating macrophages.

Fig. 5.13 shows the results of incubating SH3 DC with a fixed concentration of ^{125}I-Protein. The number of receptors, (R), were calculated from ^{125}I-IL-1 specific activities. The pattern of receptor binding after SH3-1 challenge was reproduced during the repeat SH3-2 challenge. Both ^{125}I-αm and ^{125}I-βm binding are seen to increase from 18h post challenge. Increased ^{125}I-βm binding appears to be transient, 24-48h, whereas ^{125}I-αm increases rapidly to peak over days 2-3. The binding then slowly decreases over the next 6-7 days. This pattern of IL-1α receptor expression correlates with the cell output. Unfortunately the SH3-1 66h sample, which might have been expected to show peak ^{125}I-IL-1α binding, clotted while being centrifuged through metrizamide and no DC could be salvaged.

Scatchard data on DC during the secondary response is not complete because of the dependence on cell numbers. Curves can be fitted to the data, as seen on the representative plots in Fig. 5.14, but more points are required to be accurate. Plots of data from other time points are not shown but follow a similar pattern. Estimated figures for numbers of receptors and K_d values can nevertheless give an indication of trends (see Table 5.5).

FIGURE 5.13

Sheep No.3: IL-1 Receptors Detected on Afferent Lymph Dendritic Cells in Response to Secondary Ovalbumin Challenge

Incubation of 2 x 10^5 cells with 1ng ^{125}I-IL-1

Figure 5.13

Sequential samples from the cannulated sheep SH3 were collected after challenge with 50μg OVA and isolated DC assayed for IL-1R expression. Graphs show IL-1R detected by incubating 2 x 10^5 DC with a fixed concentration of ^{125}I-IL-1. Total bound radioactivity was counted and the number of binding sites calculated from the specific activities of the labelled proteins. A control p1 preparation did not bind detectably to the DC at any stage throughout the cannulation.. The sheep was challenged twice with OVA (Ova 1 and Ova 2) and the two series of results have been superimposed. Total cell and DC output over the two cannulation periods is shown on the overlaid transparent film in order to demonstrate the co-incidence of cell counts and receptor expression. Binding sites could not be determined at 63h post Ova.1

a. Receptor expression detected by incubation with ^{125}IL-1α

b. Receptor expression detected by incubation with ^{125}IL-1β

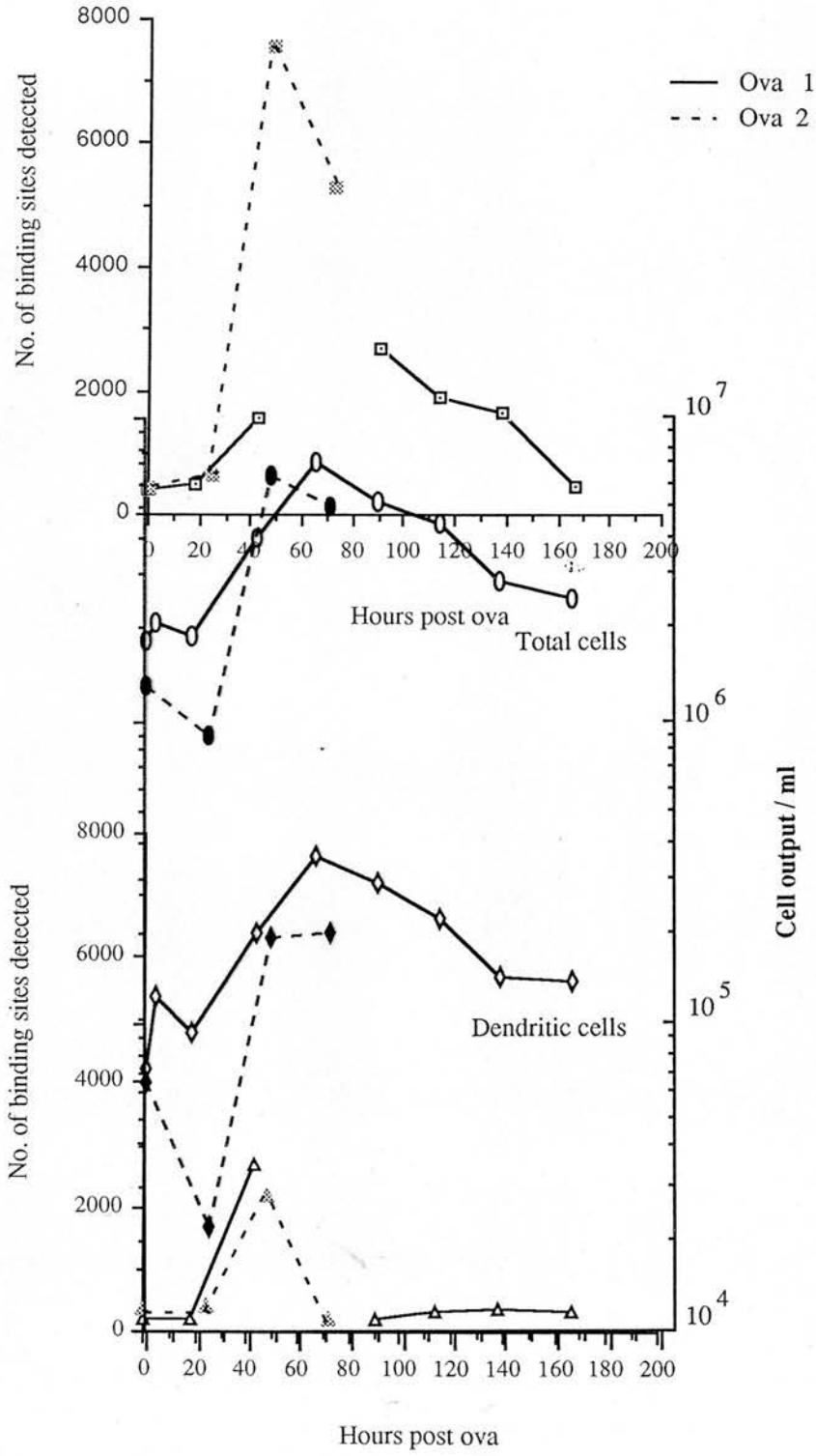
FIGURE 5.13

IL-1 Receptor Binding Sites on Afferent Lymph Dendritic Cells

Cell Output in Afferent Lymph after Secondary Ovalbumin Challenge

a. IL-1α

b. IL-1β



None of the Scatchard plots post ovalbumin are obviously bi-phasic, which may just be a consequence of the small number of IL-1 concentrations used. More importantly, all samples showing increased ^{125}I-αm binding also show reduced affinities. Of interest is that the Kd for ^{125}I-αm, which has returned to normal by day 10, increases again with increased binding at 48h and 72h after the repeat challenge. The specificity of the enhanced ^{125}I-αm binding was confirmed by cold competition using the SH3-1 114h sample, IL-1α and IL-1β being equally efficient at competing with ^{125}I-αm (>72%). Scatchard plots of IL-1α binding after OVA challenge are shown in Fig. 5.14. Plots from both challenges show similar profiles.

^{125}I-βm binding data is difficult to interpret. After secondary challenge the Scatchard plots for all samples showing resting levels of ^{125}I-βm binding are all similar, as expected. However, Scatchard plots could not be constructed for the 2d samples of either SH3-1 or SH3-2, both of which do show increased ^{125}I-IL-1β binding, indicating that the increased binding may not just represent upregulation of receptors specific for IL-1β. SH3-1 and SH3-2 also both show less than baseline binding immediately following the peak. SH3-1 binding then returned to baseline by 114h and remained at this level until re-challenged. The specificity of IL-1β binding was tested on day 4 (114h) and at this time point, ^{125}I-IL-1β was competed off efficiently by IL-1β (85%) and less so by IL-1α (53%). This information may not however be of any value with respect to the specificity of the 2 day peak binding because the 114h Scatchard plot is acceptable and shows the expected parameters.

The few results on the early response to OVA challenge which were obtained from SH1 and SH2 indicate similar trends to those detailed for SH3. An increase in bound IL-1 was detected at 4h and IL-1 binding had returned to normal by 8h (SH2), confirming a very transient early increase in receptor expression.

At no time did the irrelevant protein bind to any significant degree.

FIGURE 5.14

Scatchard Analysis of ^{125}I-IL-1α Binding to Dendritic Cells after Secondary Ovalbumin Challenge

Figure 5.14

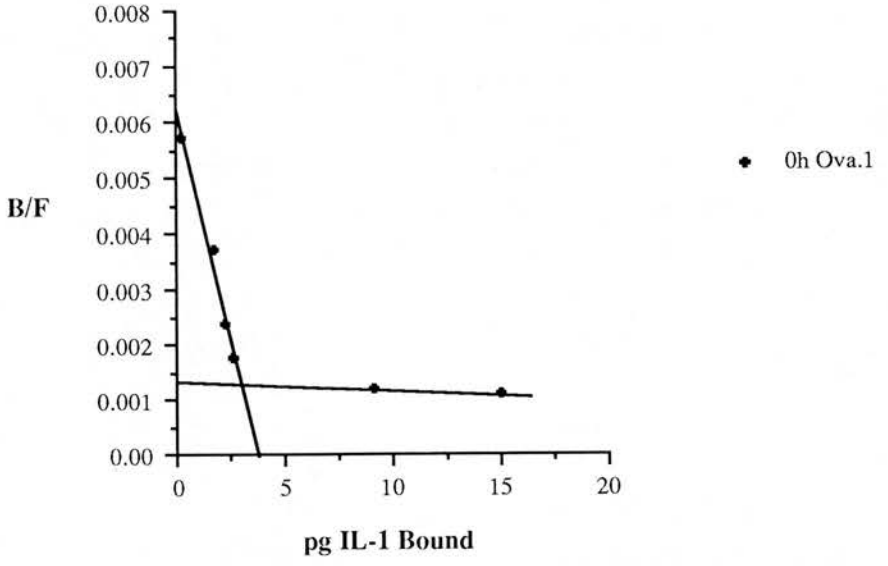
^{125}I-IL-1 binding to 2 x 10^5 DC was determined using the standard assay.

a. ^{125}I-IL-1α binding by SH-3 afferent lymph dendritic cells immediately prior to OVA challenge

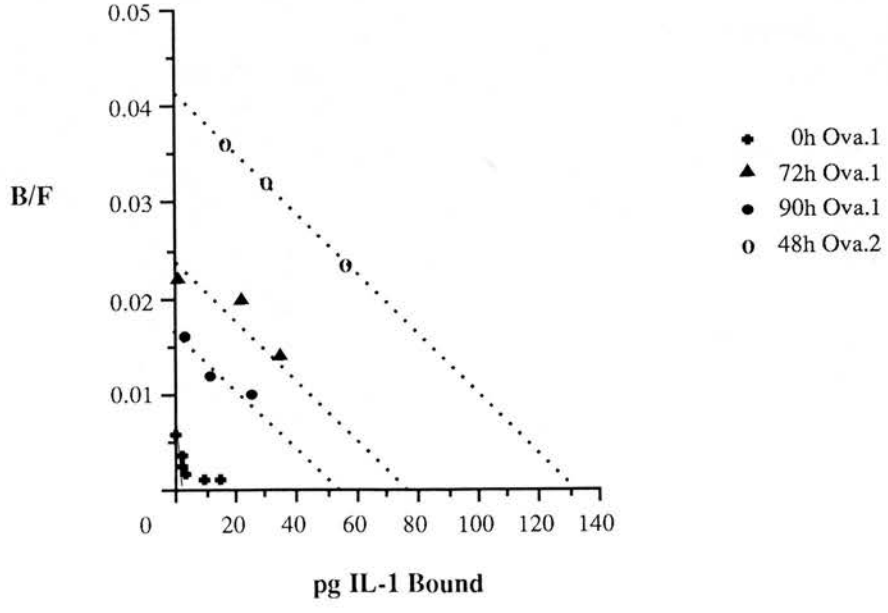
b. Data from **Fig. 5.14.a.** compared with ^{125}I-IL-1α binding at 0h, 90h after Ova.1 challenge and 48h and 72h after the repeat, (Ova.2) challenge. Hatched lines have been used for determination of receptor numbers and K_d values because of the minimal numbers of points on these plots.

FIGURE 5.14

a. ^{125}I-IL-1α Binding to SH3 Afferent Lymph Dendritic Cells Immediately Prior to Ovalbumin Challenge



b. ^{125}I-IL-1α Binding to SH3 Afferent Lymph Dendritic Cells After Secondary Challenge with 50μg Ovalbumin



5.8.3.2.2 Cytospin analysis

Between 800 and 1000 cells in consecutive fields were analysed for the presence of silver grains. Cells were only counted in areas of low background, (ie. not exceeding 20 grains over cell equivalent areas). To be positive, cells had to have more than three times the background grain count. Only a small percentage of cells displayed grains and the results are therefore quoted in two different ways in Table 5.5; (column 4) the average number of grains/+ve cell, ie. including only those cells which were positive; (column 5) grains/cell, being the total number of grains with respect to the total number of cells counted. This latter figure should relate to the Scatchard 'sites/cell' figure, ie. an overall figure not subdivided into IL-1R expression on individual cells.

By 4h after secondary challenge, a large but transient increase in the number of ^{125}I-αm positive cells was seen on cytospins but with a concommitant decrease in the number of grains on each cell. By 90h, the number of sites/cell had increased nearly twenty times, which correlated with increases in the number of positive cells and grains/cell. However, the number of grains/+ve cell was still less than at 4h. By day 10, ie. just before re-challenging with ova, grain counts and Scatchard data both showed a return to normal. Due to limited numbers of cells, no cytospin data was obtained after re-challenge.

Odd cells, have extremely heavy graining which appears to emanate from a focal point (Fig. 5.15.B.f). The pattern of grains seems to be specific and not artefactual because (i) all such heavy graining is cell associated, as demonstrated by giemsa and NSE staining, (ii) all these cells display similar patterns of grains, and (iii) no random 'hot spots' were seen on these slides. No similar patterns of binding to macrophages have been seen. These cells are seen in all IL-1α-DC samples but more are apparent at times of increased binding eg. 0.002% pre ova; 0.85% at 4h post ova; 0.1% at 43h and 0.25% at 90h. For reference, grains/cell (column 5 Table 5.5) are given both excluding and including these cells. The latter is impossible to assess accurately and can only be an underestimate, as will be understood from the photograph. Additional evidence for these cells not being artefactual comes from comparisons of the IL-1α grains/cell and sites/cell figures. For example, the figure of 9116 sites/DC at 90h post ovalbumin can be reconciled with the Mϕ data, but only if these 'extra hot' cells are included in calculations.

FIGURE 5.15

Demonstration of ^{125}I-IL-1α Binding by Individual DC after Secondary Ovalbumin Challenge

A.

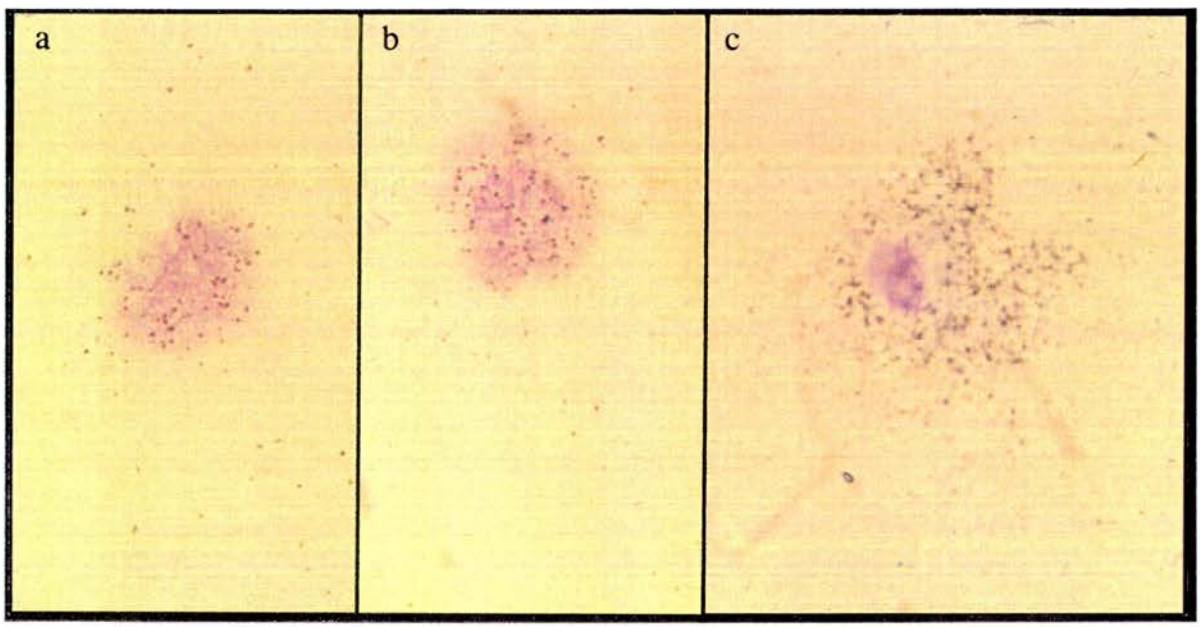
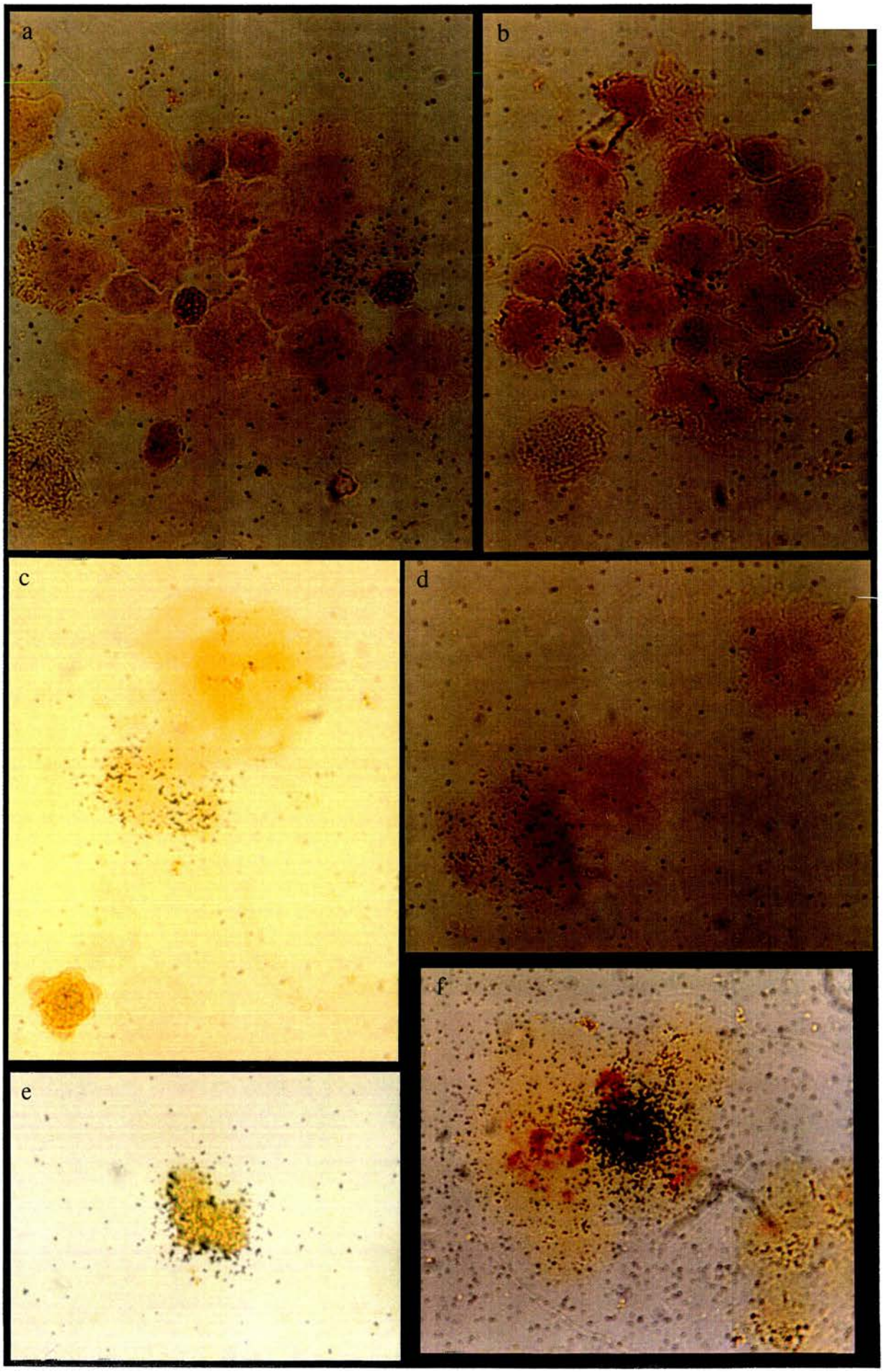


Figure 5.15
2 x 10^5 DC were incubated with 5ng ^{125}I-IL-1α under standard assay conditions.

A. Giemsa stained cells:
Cytospins were exposed with Amersham LM-1 emulsion, developed and stained.
Individual cells are shown to give an indication of variations in grain density which can be obtained.
Photographs were taken the following magnifications:
a,b (x1000); **c** (x1500).

B. Non-specific esterase stained cells:
Cytospins were stained for NSE prior to exposure.
Photographs demonstrate how individual cells within a cluster are selectively labelled.
Comparative magnifications are as follows:
a, b (x500); **c** (x1000); **d** (x780); **e** (x600); **f** (x500).

B.



Cytospin analysis of IL-1β binding at 43h (Ova-1) and 48h (Ova-2) post challenge shows no evidence of specific increased binding at these times, either in terms of the proportion of positive cells or of cell-associated grains. If anything, the number of positive cells appears to be reduced. This data confirms the lack of specific binding detected by Scatchard analysis.

5.8.3.2.3 Compilation of Scatchard and Cytospin Data

Table 5.5 shows combined cytospin and Scatchard data for IL-1 binding before and after secondary ovalbumin challenge. Macrophage binding data is included for comparison. The figures for resting lymph (1^o) are those from Table. 5.3 and were derived from a number of samples from different sheep. Data for secondary challenge (2^o) is from sheep SH3.

Scatchard analysis calculations:
Results were plotted in the accepted format of Bound/free cpm ^{125}I-IL-1 vs. pg ^{125}I-IL-1 bound. The number of receptors/cell was calculated from the x intercept and the K_d value from 1/slope (Scatchard, 1949).

IL-1β data is included but grain counts after challenge were difficult to establish and evidence for specific receptor expression for ^{125}I-IL-1β was very inconclusive.

Table 5.5

Column 1; Mo - alveolar macrophage; DC -afferent lymph dendritic cell.
Column 2; 1° - resting state; 2° - secondary challenge; Sample times are quoted as hours post ova challenge; $_{(1)}$ - first secondary challenge; $_{(2)}$ - repeat secondary challenge.
Column 3; % cells on cytospins with associated grains. 5000 cells in consecutive fields were counted.
Column 4; Average number of grains counted over individual cells on cytospins.
Column 5; The total number of grains counted over 5000 cells expressed as grains/percell. Figures are given both excluding and including the extremely heavily grained cells. The latter are given in brackets.
Column 6; Sites/cell calculated from Scatchard plots such as shown in Figs. 5.12 and 5.15.
Column 7; K_d calculated from Scatchard plots such as shown in Figs. 5.12 and 5.15.

Data in Columns 4 and 5 was derived from cytospins of 2 x 10^5 cells incubated with 5ng ^{125}I-IL-1.
Data in Columns 5 and 6 apply to total cell populations and do not take account of any individual cell data.

na - not available;
ns - potential non-specific binding, therefore unable to obtain Scatchard plot or reliable grain counts

TABLE 5.5

IL-1α Receptor Expression by Ovine Afferent Lymph Dendritic Cells Detected Before and After Ovalbumin Challenge.

Comparison with Resting Dendritic Cells and Alveolar Macrophages

Column No: 1	2	3	4	5	6	7
Cells	Sample	% +ve cells	grains/+ve cell (single cells)	grains/cell (over total cells)	sites/cell (ex Scatchard analyses)	K_d pM
IL-1αm:						
Mϕ		14	30-200	~25	2600 ± 198	56 ± 11
DC	**1°**	<0.5	41 ± 25	<0.1	510 ± 56	30 ± 4
	2° - $0_{(1)}$	<0.5	<40	<0.1	568	29
	4	2.4	64 ± 36	0.96(>7)	na	na
	43	1.2	26 ± 19	0.23(>1)	4300	168
	90	5.6	42 ± 12	2.25(>100)	9116	181
	168	<0.5	<50	<0.1	490	31
	2° - $0_{(2)}$	na	na	na	435	29
	48	na	na	na	21700	181
	72	na	na	na	13180	180
IL-1βm:						
Mϕ		24	30 to >500	~100	16500 ± 707	4.6 ± 0.5
DC	**1°**	<0.5	94 ± 43	<0.05	350 ± 145	160 ± 58
	2°-$0_{(1)}$	<0.5	<120	<0.05	344	185
	18	<0.5	<130	<0.05	360	na
	43	<0.1	<150	ns	ns	ns
	90	<0.5	<80	<0.05	74	39
	114	<0.5	<100	<0.05	385	172
	2°-$48_{(2)}$	na	na	ns	ns	ns

DISCUSSION

IL-1 is known to enhance dendritic cell function but the mechanism has not yet been elucidated. The aim of this study was to determine whether ovine afferent lymph dendritic cells express receptors for IL-1 and if so, how this expression is affected during a secondary response to ovalbumin challenge. The study was approached in such a way as to be able to determine both surface expression of IL-1R (using ^{125}I-IL-1) and co-expression of IL-1R with surface antigens known to be expressed by dendritic cells (using biotinylated IL-1 and FACS analysis). Ovine alveolar macrophages were used as control cells for two reasons; (i) Mϕ express high levels of IL-1 receptors and would therefore be a positive control for specific IL-1 binding; (ii) The presence of contaminating Mϕ in afferent lymph would have to be determined as these could bias DC binding figures.

Quantitation of IL-1R expression by binding ^{125}I-IL-1 to its cellular receptor has been successfully applied to numerous human and murine cell types (Appendix). Two studies, on bovine neutrophils and skin fibroblasts, provide the only data on IL-1R expression in ruminants (Lederer and Czuprynski, 1992, 1993).

To determine receptor expression using labelled ligand, it is essential to have purified protein which retains biological activity after labelling. Chloramine T and Bolton and Hunter methods have been successfully used for iodinating human rIL-1 (Dower et al., 1985, Fuhlbrigge et al., 1988) and very recently lactoperoxidase iodination of bovine rIL-1 was reported (Lederer and Czuprynski, 1993). The more gentle Iodobead method used for labelling ovine rIL-1 was equally efficient and reproducible, yielding specific activities in the range 0.7–1 x 10^6 cpm/pmol without any significant reduction in bioactivity. Biotinylation of IL-1 on the other hand yielded material which could not be used for receptor studies. The proteins labelled efficiently but only retained minimal assayable activity. In addition, biotinylated IL-1β did not compete with ^{125}I-IL-1β binding to macrophages whereas unlabelled material did. From the data available on residues involved in human and murine IL-1β activity and receptor binding (see Chapter 1, Sect. 1.11.1.6), some of the equivalent ovine residues, Gln-4, Gln-24, Lys-93 and Lys-102, could potentially be biotinylated with resultant inhibition of IL-1β receptor interaction. Recently biotinylation has also been shown to reduce the activity of recombinant ovine TNFα to unacceptable levels (B. Lund, personal communication). Because of the DC subpopulations present in afferent lymph, fluorescently labelled IL-1 would be useful for determining co-expression of IL-1R with various other markers using two colour

FACS analysis. Use of FITC, which binds to the same residues but is much smaller than biotin, could cause less interference with IL-1R binding and may be worth future investigation.

FACS analysis of macrophage, DC and lymphocyte populations confirmed previous data (Bujdoso et al., 1989) that resting afferent lymph DC express minimal $CD4^+$, intermediate levels of CD1 (VPM5) and strong MHC Class II (VPM54) surface antigens. In comparison, ovine Mϕ do not express VPM5 and Class II expression is two orders of magnitude less than on DC. The purity of DC preparations was estimated at ≥80% by FACS, the remaining 20% of cells being lymphocytes. Cytochemical staining for non-specific esterases confirmed that contaminating macrophages were present only in very low numbers. Peripheral blood DC express similar markers to afferent lymph DC (Caux et al., 1992; Thomas et al., 1993) and the presence of PB DC could not therefore be ruled out. Staining for acetylcholinesterase which, in sheep, is specific for LC or LC derived cells, also could give no reliable indication of the presence of PB DC because it is not known to what extent AChe activity varies with maturation of LC to DC. The presence of few erythrocytes was therefore taken as an indication of minimal blood contamination.

The resting rate of DC influx into the sheep node was about 5 x 10^5/h, comparable to rat mesenteric and rabbit popliteal lymph nodes at 1 x 10^5 and 2 x 10^5 DC/h respectively (Pugh et al., 1983; Kelly et al., 1970). This increased to >1.3 x 10^6/h over the first 4h and to >3.8 x 10^6/h between 24h and 72h after secondary challenge but without any significant alteration in the proportion of NSE or AChe positive DC.

For IL-1 binding assays precautions were taken with respect to incubation conditions. Because IL-1/IL-1R complexes can be rapidly internalised and IL-1 can also affect expression of its own receptor (Matsushima et al., 1986b; Akahoshi et al., 1988; Takii et al., 1992; Lacey et al., 1990; Shieh et al., 1990; Arend et al., 1991; McMahan et al., 1991), Na azide was always added to inhibit this internalisation and signal transduction (Uhl et al., 1989; Horuk et al., 1987). In addition, all incubations were done at 4°C because thymocyte proliferation assays had indicated that incubation at 37°C could reduce the biological activity of ovine IL-1 (See sect. 4.5.1).

The majority of published ligand binding studies using cell lines have relied on the use of >10^6 cells per sample, which allows work well above the limits detection, hence increasing accuracy. Despite having to use low cell numbers (2x10^5) in the current study, the intra-assay variability for IL-1 binding to *ex*

vivo afferent lymph cells was low, although some differences between animals were seen.

In considering IL-1R expression on afferent lymph DC, two different but complementary representations have been taken into account; (i) overall expression with respect to the whole DC population, ie. Scatchard and sites/cell data; (ii) expression by individual cells as visualised on cytospins. Scatchard sites/cell exhibit a positive correlation with cytospin grains/cell but the efficiency of autoradiographic detection is low and long exposure times are required.

Using the ^{125}I-IL-1 binding assay, resting ovine afferent lymph DC, *ex vivo*, have been show to express ~500 receptors (K_d 30pM) for IL-1α and ~350 receptors (K_d 160pM) for IL-1β. IL-1R expression has also been shown to be upregulated during the inflammatory response following secondary ovalbumin challenge. Kampgen et al., (1992) recently presented data on cultured murine LC and DC which expressed $\geq$500 and $\geq$100 IL-1R per cell respectively (Kd<100pM), but I am not aware of any other studies of IL-1 binding by dendritic cells in the peripheral immune system.

The response of IL-1R expression by DC after secondary ovalbumin challenge to sheep SH3 was greater for IL-1α than for IL-1β (Fig. 5.14 and Table 5.5). A transient but significant increase in IL-1α but not IL-1β binding is seen on cytospins at 4h post ovalbumin challenge. Unfortunately there were not enough cells for multiple assays and Scatchard binding data was not obtained at this time. IL-1α and IL-1β binding both increase from about 24h post challenge and maximal expression occurs between 43h and 72h post challenge. Cytospin data correlate positively with this, showing increases in the precentage of positive cells and in grains/cell. Figures for binding of 1ng ^{125}I-IL-1 to 2 x 10^5 DC show that IL-1α binding has been enhanced nearly eighteen-fold and IL-1β at least ten-fold at 43-72h. Equilibrium binding data for this time point reveals a forty-fold increase to $\leq$21700 IL-1α receptors/cell but this figure is based on an incomplete Scatchard plot (Fig. 5.15.b) and may well be an overestimate. Resting level IL-1β binding appears to be specific but Scatchard data on the increased IL-1β binding is difficult to interpret and may represent some non-specific binding, a conclusion supported by cytospin data. In addition, the repeat Ova.2 challenge would be expected to produce a greater response to ovalbumin than after the first Ova.1 challenge. This was clearly seen with IL-1α binding to DC but IL-1β binding was no greater after the re-challenge. There is therefore no clear evidence that receptors for IL-1β are specifically upregulated during a secondary response to antigen.

These results support published evidence indicating that both interdigitating thymic reticulum cell and Langerhans' cell function may be dependent on IL-1α but not IL-1β (Ruco et al., 1990). Upregulation of the number of receptors for IL-1α appears to correlate inversely with their affinity. The K_d value increases from 29 ± 1pM on resting cells to 173 ± 11pM at high IL-1R expression and returns to the lower figure as receptor expression decreases. Similar data on IL-1R upregulation and affinity (but for IL-1β in this case), has been obtained from *in vitro* treatment of two human B cell lines, STS25 and H7, with dexamethasone (Stoppacciaro et al., 1991). It is not clear from my data whether DC IL-1R upregulation involves high or low affinity sites but this may be a reflection of the non-homogeneous nature of the DC population. Individual cell data suggests that the few extremely heavily grained cells could be the main contributors to the altered K_d values.

Dissociation kinetics were not calculated but the higher affinity of the receptor for IL-1α is corroborated by the finding that only 9% of bound ^{125}I-IL-1α but 25% of bound ^{125}I-IL-1β dissociated from DC during overnight incubation at 4°C. There should be no internalisation of the ligand at this temperature in the presence of 0.1% Na azide. Dissociation of bound ^{125}I-IL-1β from bovine and porcine fibroblasts is in the same range, irrespective of the presence or absence of cold IL-1β. Rapid initial dissociation becomes gradual dissociation and has more or less reached equilibrium at 30% dissociated material by 6h. Greater dissociation is seen at 37°C (Bird et al., 1987, Lederer and Czuprynski, 1993).

Analysis of IL-1R expression at a single cell level shows that ^{125}I-IL-1α binding is heterogeneous. Only a very small proportion of resting DC bind IL-1α and OVA challenge causes an increase both in the number of positive cells and in the grains detected on each cell. There is also an increase in the number of cells showing extremely large numbers of grains. It is possible that the latter are displaying non-specific binding of ^{125}I-IL-1α but the sites/cell figures and the consistent patterning of grains, in the form of a halo around the cells which appears to emanate from a focal point (Fig. 5.18), would argue against this.

In view of the diversity of DC subtypes within afferent lymph, it is possible that if the action of IL-1α is transitory, IL-1R expression will only be detectable at certain stages of differentiation or activation of the cells. On a morphological basis, the only cells with associated grains are either dendritic in nature, as opposed to veiled, non-granular DC which stain weakly for non-specific esterase or the occasional smaller DC with reticular staining (types c and d respectively, Sect 5.3.3.3). The type of staining exhibited by cells with an extremely high

density of grains is very difficult to determine, but seems to be stronger than that of cells with fewer grains and more like the types b or f staining described in Sect. 5.3.3.3 (Fig. 5.6). Whether these cells represent either discrete stages in DC maturation or distinct phenotypic subgroups within the population would have to be determined by dual staining with fluorescent markers. Too few cells were available to be able to compare these cells with those positive for AChe.

IL-1RI bound IL-1α (Grenfell et al., 1989) and IL-1β (Qwarnstrom et al., 1988; Falk et al., 1989; Grenfell et al., 1991) can be internalised, although this may not be essential for activity. The internalised IL-1 is undegraded, retains biological activity and accumulates both on nuclei and in lysosomes. Late endosomes within DC are now known to be involved in antigen processing (Levine and Chain, 1992) and Arkema et al (1991) have shown clustering of Class II positive vesicles and lysosomes in the juxtanuclear area of human DC. This is also the region which specifically reacts with the human anti-CD68 (EBM11) and murine ED1 myeloid markers (Betjes et al., 1991; Beelen et al., 1992). Although ^{125}I-IL-1 should not have been internalised to any extent under the conditions used, it is interesting to speculate on whether this could also be the area from which the higher intensity graining emanates hence indicating concentration of IL-1 receptors in specific areas. The type of concentration of receptors seen in the study being reported here has not, to my knowledge, been described for other cells, although clustering of IL-1 receptors into focal adhesions on human fibroblasts has been described (Dower et al., 1990). This latter study visualised grains by electron microscopy and therefore represent a different order of detection. In view of the highly specialised nature of the DC it is possible that specialised expression of membrane proteins has developed.

Comparison with IL-1 receptor/cell expression on alveolar macrophages (IL-1α ±2600; IL-1β ±17000) shows that resting DC express minimal numbers of receptors (IL-1α ±500; IL-1β ±350). Activated DC can express nearly ten-fold more receptors than Mϕ for IL-1α but fewer receptors than Mϕ for IL-1β. These figures are assessed with the proviso that Mϕ activation, either by anaesthetic or by manipulation at the time of cell collection, could potentially increase IL-1R expression in which case the figures obtained are not those of resting Mϕ. IL-1β binding to ovine alveolar macrophages is comparable to IL-1β binding to human RAJI/H7 B cells, ie. 24% ovine Mϕ expressing 16500 IL-1R/cell and 15% resting RAJI cells expressing 15524 IL-1R/cell which is upregulated to 28% expressing 45000 IL-1R/cell when stimulated (Stoppacciaro et al., 1991). Irrespective of the state of activation of the macrophages, it is obvious that activated DC bind IL-1α preferentially whereas Mϕ bind IL-1β preferentially. In addition, both IL-1α and IL-1β could compete for binding to DC but, although

IL-1β competed with IL-1α binding to Mϕ, IL-1α did not effectively compete with IL-1β binding.

Because of the small numbers of lymph DC which express detectable IL-1R, cross-linking or ligand blotting techniques were not sensitive enough to allow determination of the size of the receptor. Pulse labelling experiments could be used to determine the kinetics of internalisation of bound ligand, IL-1/IL-1RI complexes being rapidly internalised and IL-1/IL-1RII not being internalised to any great extent (Horuk et al., 1987). Alternatively PCR or *in situ* PCR with primers derived from human IL-1R sequences might be useful, depending on sequence homologies. A number of pieces of circumstantial evidence do however point to the receptor type being IL-1RI rather than the IL-1RII which is expressed by macrophages. IL-1RI and IL-1RII differ in their IL-1 binding potential in a number of ways. Cells expressing IL-1RI reach binding equilibrium rapidly, 1-2h as opposed to 2-4h for IL-1RII. IL-1RI binds IL-1α better than IL-1β. IL-1RII on the other hand binds IL-1β to a much greater extent than IL-1α and certain B lymphoma lines show exclusive IL-1β binding (Dower et al., 1986). IL-1α and IL-1β can compete with each other for IL-1RI whereas IL-1α does not compete off IL-1β from IL-1RII. All of the above criteria for IL-1RI binding are exhibited by ovine afferent lymph DC and point to expression of the type I receptor.

Preliminary cross-linking results from cultured murine LC/DC have shown the IL-1R to be ±80kD and it has been suggested that this, together with a K_d of <100pM, is indicative of IL-1RI expression (Kampgen et al., 1992). The sizes of both receptors can vary with cell source ie. IL-1RI 80-100kD and IL-1RII 60-80kD, and K_d values are equally variable, so that it is in no way certain that the murine data does in fact represent a type I receptor. Ovine alveolar macrophage IL-1 binding data, in contrast to the DC data, shows IL-1RII receptor binding characteristics, as would be expected. If DC do indeed express the type I receptor, this would indicate a lineage distinct from that of macrophages. DC express markers indicating a myeloid origin (eg. CD13, CD33, CD34) and it has been suggested that LC derived DC, peripheral blood DC and Mϕ are all ultimately bone marrow derived, possibly from a common progenitor, but it is not known at what point separate development would be initiated (Caux et al., 1992; Romani et al., 1989b; Thomas et al., 1993). That other bone marrow derived cells, B cells and PMN, also express the type II receptor makes expression of the type I receptor by DC seem unlikely. In addition, there is evidence of rapid binding of rhIL-1α to a PMN receptor of about 70kD, with subsequent rapid internalisation at 37°C (Rhyne et al., 1988) demonstrating that bone marrow derived cells can express IL-1 receptors with type I characteristics.

The cytokines IL-4 and IFNγ can each elicit different responses from DC and Mϕ (Chin et al., 1990,1991). If different signal transduction pathways can be operative in the two cell types, this could also be the case for IL-1. It is conceivable that myeloid precursors could have the potential for expressing both types of IL-1R and that functional selection determines which is ultimately expressed.

In conclusion, afferent lymphocytes express no detectable receptors for IL-1β and only low levels of receptors for IL-1α, which may even decrease after secondary antigen challenge. Dendritic cells have been shown to express IL-1 receptors which are specifically upregulated with respect to IL-1α following secondary ovalbumin challenge. This upregulation appears to occur slightly in advance of increased MHC class II expression and to co-incide with increased CD1 expression. No specific upregulation of receptors for IL-1β was apparent. As large quantities of IL-1α are produced constitutively from keratinocytes, it was initially thought that IL-1α rather than IL-1β might be the more important for LC/DC function. The IL-1 receptor data confirms that this is the case after secondary antigen challenge but resting LC/DC display only a slight preference for IL-1α. The implications of the dendritic cell IL-1 binding data will be discussed in Chapter 6.

CHAPTER 6

DISCUSSION

6.1 General Considerations and Sequence Conservation within the IL-1 Family

This project was initiated because of the central role of IL-1 in the pathology of immunity, inflammation and infection. Little was known about any of the ovine cytokines and it was considered important to be able to monitor alterations in IL-1 production and activity caused by various disease states in the sheep. If the involvement of ovine IL-1, or certain aspects of it's actions, could be understood, the potential for therapeutic intervention could become a subsequent consideration.

No useful reagents were available with which to study ovine IL-1. Human and murine IL-1s had been cloned and there were antibodies to the expressed proteins but these were of little use in ovine systems because of low species cross-reactivity. The prerequisites were therefore twofold; (i) cloned IL-1α and IL-1β cDNAs for use as molecular probes and (ii) expressed recombinant IL-1 proteins for use in IL-1 receptor studies, antiserum production and the development of specific ovine IL-1 assays. The project was not intended to encompass assay development.

In the process of successfully cloning and expressing ovine IL-1 a number of interesting factors became apparent. Amplification of cDNA sequences by PCR although allowing rapid cloning, did introduce errors, either as a result of infidelity due to the *Taq* polymerase enzyme or mispriming during anchored PCR reactions or via chimeric products, all of which reduce the efficiency of the system. Three potential IL1β polymorphisms but no IL-1α polymorphisms were detected. The ovine IL-1α and IL-1β proprotein/mature protein sequences were found to be 97/98% and 95/96% similar respectively to their bovine counterparts. The comparatively low homology found between the mature ovine IL-1s and the human (IL-1α, 69%; IL-1β, 64%) or murine (IL-1α, 54%; IL-1β, 59%) mature proteins, substantiated the low species cross-reactivities mentioned above. In confirmation, the expressed ovine IL-1 proteins also exhibited species specificity in assays on murine tissue. A similar species barrier has been observed for the ovine cytokines TNFα and GM-CSF (Green et al., 1993;

McInnes and Haig, 1991) and Lederer and Czyprynski (1989b) have reported a species preference of bovine thymocytes and fibroblasts for bovine IL-1. This specificity is of interest because there is little evidence of other IL-1 species specificity using a variety of mammals and even reptiles and fish. It has however been suggested that species specificity can play a role in some biological responses. One report on IL-1 triggered release of ACTH from rats shows that rat and human IL-1β are equally efficient whereas rat IL-1α is 10 times more potent than human IL-1α (NaitoY et al., 1989) and it has been postulated that cells which express primarily the type II receptor may be more species restricted than cells bearing the type I receptor (Dinarello, 1991). The ovine data indicates a clear species specificity for IL-1 biological activity ellicited through the type I receptor. The apparently more distinct species preference exhibited by ovine/bovine IL-1 as compared to other species will be analysed in relation to sequence alignments in Sect. 6.1.1.3.

6.1.1 Assessment of Conserved Motifs at the Primary Structure Level

All known IL-1 cDNA sequence translations are aligned in Table 6.1. The human IL-1ra sequence is included for reference and has been related to human IL-1β with respect to conserved residues. Protein sizes and species identities relative to the respective ovine proteins have also been included. Dots in the ovine IL-1β sequence indicate conserved residues with reference to ovine IL-1α. For all other species, dots indicate conserved residues with referecnce to the equivalent ovine IL-1 sequence. Twelve common motifs within β-strands of the three tertiary structures (Eisenberg et al., 1991) are indicated by horizontal bars and the three potentially polymorphic ovine IL-1β residues which were detected in the current study are shown. 33 residues are conserved across IL-1α and IL-1β and 12 are conserved across all members of the IL-1 family. Potential glycosylation sites are present in all sequences.

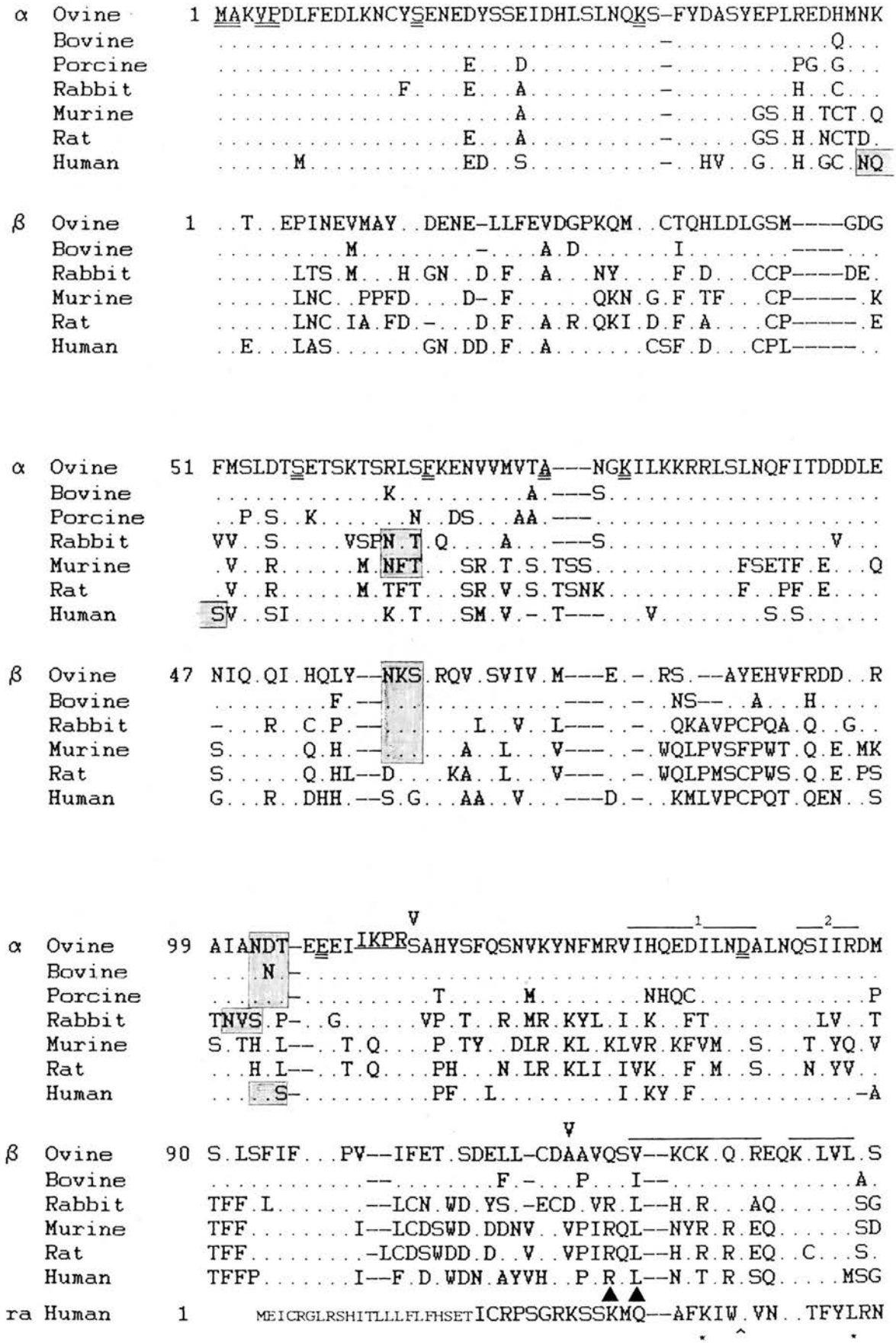
Patterns emerging from these alignments will be discussed first and individual residues addressed subsequently with reference to the tertiary structure of the proteins. The N-terminal region of IL-1α shows minimal variation. The IL-1β N-terminal sequence is far more variable but the C-terminal sequences of both proteins are similarly variable. Both IL-1α and IL-1β exihibit stretches of low conservation at the N-termini of their mature protein sequences which has been thought to be of relevance with respect to the different activities attributable to these two proteins. The importance of conserved motifs has been extensively reviewed and the most important features have been described in Chapter 1. Only features relevant to ovine IL-1 activity will therefore be discussed here.

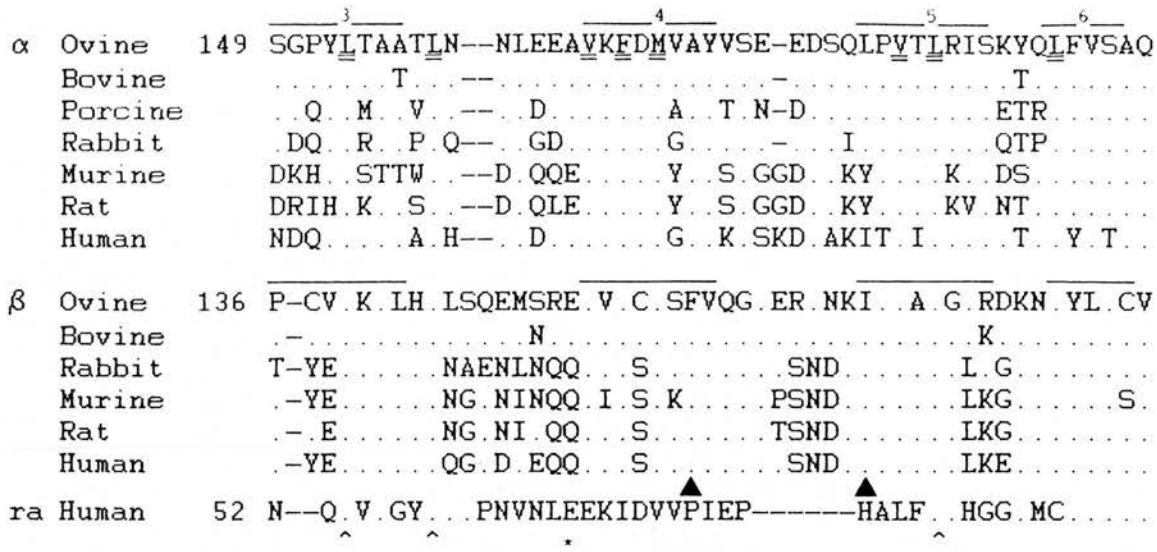
Figure 6.1

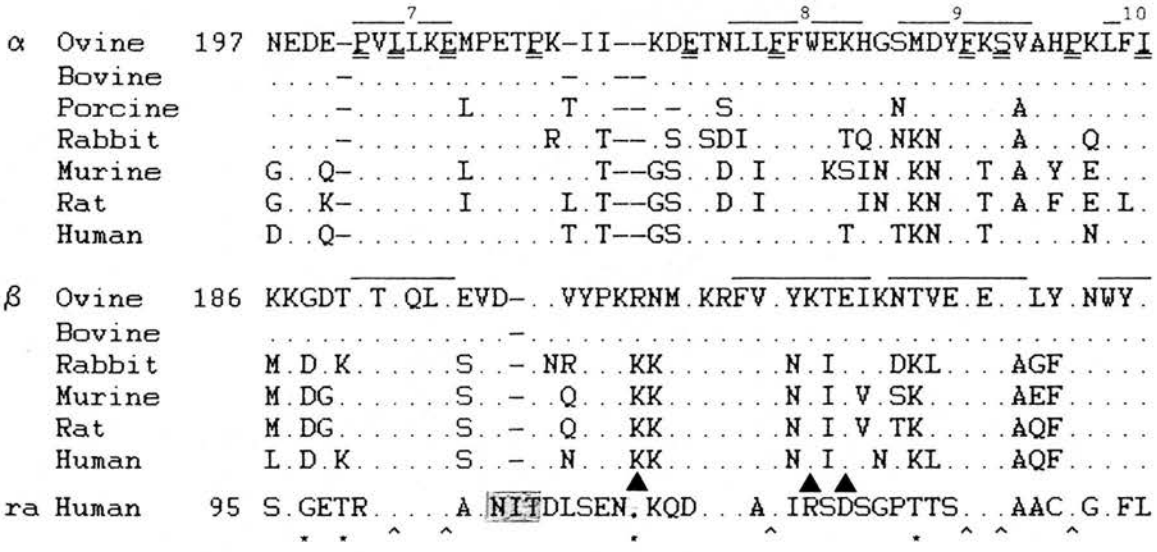
All known IL-1 amino acid sequences are aligned compared to the respective ovine sequences. Ovine IL-1β has also been related to ovine IL-1α with respect to conserved residues. The human IL-1ra sequence has been related to human IL-1β with respect to conserved residues. Twelve common motifs within β-strands of the three tertiary structures are indicated by horizontal bars over the sequences and potential glycosylation sites are shaded. The IL-1ra secretory sequence is shown in small size capital letters.

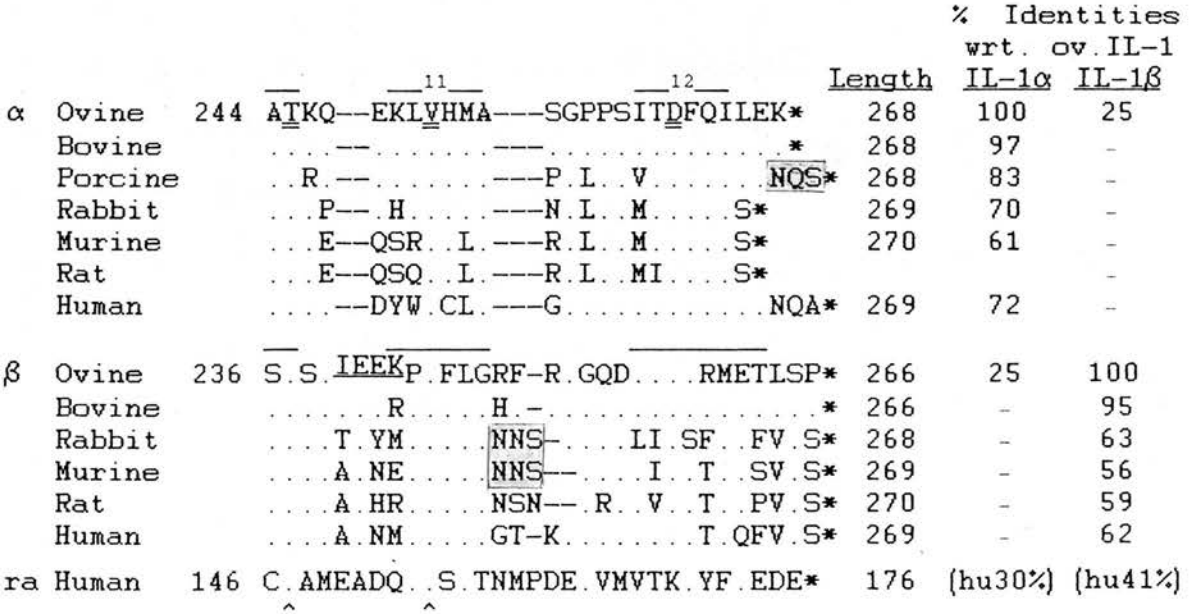
- V - start position of mature proteins
- (x) - potentially polymorphic IL-1β residues
- X - residues conserved across all IL-1 sequences
- ^ - residues conserved across all IL-1 and IL-1ra
- * - residues conserved between ovine IL-1β and human IL-1ra
- ▲ - residues forming putative discontinuous IL-1β binding site for IL-1RI
- $x^{\underline{XXXX}}x$ - Putative Factor Xa recognition site

FIGURE 6.1 Interleukin-1 Family Amino Acid Alignments




α Ovine 149 SGPYLTAATLN--NLEEAVKFDMVAYVSE-EDSQLPVTLRISKYQLFVSAQ
Bovine
Porcine
Rabbit
Murine
Rat
Human
β Ovine 136 P-CV.K.LH.LSQEMSRE.V.C.SFVQG.ER.NKI..A.G.RDKN.YL.CV
Bovine
Rabbit
Murine
Rat
Human
ra Human 52 N--Q.V.GY..PNVNLEEKIDVVPIEP------HALF..HGG.MC.....


α Ovine 197 NEDE-PVLLKEMPETPK-II--KDETNLLFFWEKHGSMDYFKSVAHPKLFI
Bovine
Porcine
Rabbit
Murine
Rat
Human
β Ovine 186 KKGDT.T.QL.EVD-..VYPKRNM.KRFV.YKTEIKNTVE.E..LY.NWY.
Bovine
Rabbit
Murine
Rat
Human
ra Human 95 S.GETR....A.NITDLSEN.KQD..A.IRSDSGPTTS..AAC.G.FL


% Identities wrt. ov.IL-1
Length IL-1α IL-1β
α Ovine 244 ATKQ--EKLVHMA---SGPPSITDFQILEK* 268 100 25
Bovine 268 97 -
Porcine 268 83 -
Rabbit 269 70 -
Murine 270 61 -
Rat -
Human 269 72 -
β Ovine 236 S.S.IEEKP.FLGRF-R.GQD....RMETLSP* 266 25 100
Bovine 266 - 95
Rabbit 268 - 63
Murine 269 - 56
Rat 270 - 59
Human 269 - 62
ra Human 146 C.AMEADQ..S.TNMPDE.VMVTK.YF.EDE* 176 (hu30%) (hu41%)

6.1.1.1 Processing of IL-1α

The IL-1α nucleotide sequence GKILKKRRLS (ovine residues 78-87) is conserved across all species, except for a human Val for Leu-81 substitution. The tetrabasic KKRR, a trypsin digestion site, was originally proposed as a potential primary processing site with the resultant molecule being further digested by other enzymes to yield the mature IL-1α (Lomedico et al., 1984). It was subsequently shown, however, that the murine IL-1α proprotein, which is phosphorylated at the adjacent Ser, is trypsin insensitive and that this phosphorylation may inhibit digestion by trypsin. Phosphorylation may be used as a control mechanism because there is evidence that unstimulated macrophages do not phosphorylate human IL-1α and this proprotein can be trypsin digested, hence leading to a disposal pathway, whereas the proprotein from stimulated Mϕs is phosphorylated and appears to be protected (Beuscher et al., 1988). This Ser is conserved in all species and is the only phosphorylated amino acid to have been detected in the IL-1 proteins. Assuming that ovine IL-1α would be subject to the same proteolytic restriction, α-Ser-87 is likely to be phosphorylated.

By analogy with other species the amino terminus of the bovine IL-1α mature protein has been presumed to be either α-Ser-120 (Maliszewski et al., 1988) or α-Ser-113 (Leong et al., 1988). Amino acids α-105–114 are highly conserved, which might indicate a protease recognition site including Ser-113. Although various means for processing IL-1α to its mature form have been postulated (see Chapter 1, Sect. 1.4), it is of interest that during this study, activated Coagulation Factor Xa appeared to cleave ovine IL-1α between Arg-112 and Ser-113. Although this has not yet been confirmed by N-terminal analysis, no other FXa recognition sites are present in IL-1α and non-specific cleavage has been ruled out. From work in our laboratory, the minimal sequence requirement for a FXa recognition site has been found to be IxxR and FXa would therefore be expected to recognise murine IQPR as well as the IKPR motif found in all other species. Jones and Geczy (1990) have demonstrated FXa-like activity on the surface of LPS stimulated guinea-pig macrophages and found that FXa and thrombin greatly induce IL-1 activity in macrophages stimulated by suboptimal concentrations of LPS. It was proposed that this could be either via direct action on IL-1 or via the action of FXa converting prothrombin to thrombin. It appears from the ovine results that FXa can act directly on IL-1α and may therefore be one of the natural processing enzymes. Sequencing of murine and human IL-1α has in fact revealed a heterogeneous population of amino termini for mature IL-1α all of which exhibit equivalent activities (Lomedico et al., 1984; March et al., 1986; Van Damme et al., 1985; DeChiara et al., 1986). The activities of ovine rIL-1α possessing either amino terminus, ie. either specifically expressed to start

at α-Ser-120 or cleaved by FXa, presumably at α-Ser-113, were both found to be comparable to other species. Because FXa had to be used to cleave IL-1 from the expressed IL-1αp:p1 fusion protein, the complete IL-1α proprotein was not available for comparison.

6.1.1.2 Processing of IL-1β

In common with human and murine IL-1β, ovine IL-1β contains the Asp-113:Hydrophobic-114 motif and is therefore probably also processed to its mature form by the specific ICE enzyme (Sleath et al., 1990). The importance of this processing has been emphasised by the finding that cowpox virus encodes a highly specific ICE inhibitor which is necessary for the virus to suppress the host inflammatory response (RayCA et al., 1992). Substrate inhibitors specific for ICE are thought to regulate macrophage output of mature IL-1β (Thornberry et al., 1992). Like IL-1α, IL-1β can be processed by any one of several proteases, such as elastase and cathepsin G, to yield mature proteins with different amino termini (Hazuda et al., 1991) but unlike IL-1α, most of these IL-1β proteins have been found to have reduced biological activity (Black et al., 1988; Mituzani et al., 1991b). Human mast cell chymase which is prevalent in the skin, generates biologically active IL-1β with N-terminal hβ-Val-114 rather than hβ-Ala-117 (Mizutani et al., 1991b). Neither ovine nor bovine IL-1β contain an equivalent site which might be susceptible to chymotryptic cleavage and it would be interesting to determine whether the granule-specific sheep mast cell proteinase (Huntley et al., 1992) can process IL-1βp produced by ovine keratinocytes.

6.1.1.3 Sequence Conservation and Species Specificity

IL-1α has been calculated to be diverging at a greater rate than IL-1β (Clark et al., 1986; Eisenberg et al., 1991), and this certainly seems to be the case for human, rabbit, rat and murine IL-1 (see Table 3.4 and Fig. 6.1). If, however, one compares identities with reference to the ovine sequences, ovine/bovine IL-1β seems to have diverged from the rest to a greater extent than ovine/bovine IL-1α. On an individual residue basis, the IL-1β alignment shows a striking pattern of conservation, residues frequently being seen to be conserved across human, murine, rabbit and rat but not ovine and bovine IL-1β sequences. This phenomenon is most marked within the mature IL-1β sequence but is also apparent to a lesser extent in the N-terminal proprotein sequence. Most of the differences between the 'human group' and 'ovine group' sequences represent conservative substitutions but may nevertheless contribute to the marked differences in species specificity exhibited by the two groups which were

mentioned at the beginning of this chapter. Interestingly, at four of these positions, β-Glu-152, β-Gly-188, β-Arg–206 and β-Thr-222, it is the ovine residue which appears in the human IL-1ra. This pattern of conservation relative to the ovine/bovine sequences is also apparent in IL-1α although it is not as obvious because of the higher mutation rate within the human, rat, rabbit and murine IL-1α sequences.

6.1.1.4 Conservation and the Receptor Antagonist

The IL-1 receptor antagonist does not contain sequences present at the presumed start positions of either of the IL-1 mature proteins, ie. DA in IL-1β and IKPRS in IL-1α, further evidence for the specificity of these sites.

IL-1ra competes with both IL-1α and IL-1β for binding to both IL-1RI and IL-1RII. Because IL-1ra binds to the IL-1 receptors without eliciting a biological response, analysis of residues conserved across the mature IL-1 proteins and the antagonist could be expected to yield information on which amino acids might be important for receptor binding and/or activity. Twelve residues are conserved across all members of the IL-1 family but otherwise residues conserved between hIL-1β and hIL-1ra are different to those conserved between hIL-1α and hIL-1ra. A few IL-1ra residues are equivalent to ovine rather than human IL-1β and are indicated on Fig. 6.1 (*). The potential importance of these will be discussed in Sect. 6.1.2. At only one IL-1α residue, α-Val-170, is the IL-ra residue equivalent to ovine rather than human IL-1α, which may implicate this residue in the observed IL-1α species specificity.

Examination of the residues emphasises how cautious one must be in drawing conclusions from primary structure data. For example, one third of the IL-1ra/hIL-1β conserved residues are within the β-173-204 region thought to be involved in activity not binding. In addition, far more residues are conserved than would be expected to be necessary for receptor binding. By comparison, only ~5% of total residues are required for C5a or human growth hormone binding to their respective receptors (Mollison et al., 1989; Cunningham et al., 1990). Some of the residues are required to conserve the general shape/structure of the protein although not in actual receptor contact themselves. It also has to be borne in mind that there is likely to be some chance conservation of the sequence of the primitive gene prior to gene duplication.

A more appropriate analysis might be of residues conserved across IL-1 but not the antagonist. Of the twentytwo amino acids conserved across the mature IL-1s, eleven are not present in the antagonist. These latter residues are distributed

throughout the tertiary structure (see Fig. 6.2.b). Only one of these eleven IL-1α residues has so far been mutated, hα-Met-168 to Leu which resulted in some loss of LAF and 50% loss of A375 proliferative activities. This loss of activity may be due to structural alterations (see Sect. 6.1.2.5). Seven of the eleven IL-1β residues are within regions supposedly important for activity but not binding. Preliminary evidence has been presented that IL-1ra binding to IL-1RI may not be identical to either IL-1α or IL-1β binding (Arend et al., 1992) but the actual importance of any of these residues has yet to be established.

6.1.2 Assessment of IL-1 Conservation at the Tertiary Structure Level – Possible Contribution of Individual Residues to Species Specificity

Although there is only 25% homology between IL-1α and IL-1β amino acid sequences, and 41% homology between IL-1β and IL-1ra, crystallographic and nuclear magnetic resonance studies have shown their folding patterns and tertiary structures to be very similar, each being in the form of a trigonal pyramid made up of 12 antiparallel strands (Graves et al., 1990; Clore et al., 1990; 1991a; 1991b; Labriola-Tompkins et al., 1991; Eriksson et al., 1991; Zhu et al., 1991; Eisenberg et al., 1991; Arend et al., 1991; Stockman et al., 1992; Veerapandian et al., 1992).

Figure 6.2 has been assembled using crystallographic co-ordinates of the hIL-1β mature protein as determined by Veerapandian et al., 1992. IL-1α co-ordinates were not available and this structure will therefore be used to illustrate the positions of residues of interest with respect to both proteins, with the proviso that IL-1α and IL-1β folding patterns may not be identical. Although sequence alignments show exterior residues to be much less conserved than interior residues (Priestle et al., 1989 and Fig. 6.2.b), certain exterior residues proposed as being important for the generation of human or murine biological responses do appear to be well conserved. These residues have been highlighted in Fig. 6.2.a in order to assess their potential importance with respect to ovine IL-1 activity and will be referred to as appropriate. Residues which are not conserved in the ovine sequences are identified with an asterisk. Throughout this thesis, amino acids have been numbered from the start of the IL-1 proproteins, ie. Met-1. Because of the enumeration of the X-ray co-ordinates, residues in Fig. 6.2 are numbered as from Ala-1, the start of the IL-1β mature protein. In the discussion that follows, the equivalent ovine proprotein residue numbers will therefore be given in brackets in order to relate these residues to the alignments shown in Fig. 6.1. Only residues of relevance to the activities of ovine IL-1 will be discussed in detail.

FIGURE 6.2

Tertiary Structure of Human IL-1β

These figures have been compiled from the crystallographic co-ordinates of the hrIL-1β mature protein determined by Veerapandian et al., (1992). The ribbons represent the backbone of the molecule and white arrows indicate the N and C termini of the molecule. Highlighted residues within these representations of the IL-1β monomer are numbered as from Ala-1, the start of mature IL-1β. These residues need to be assessed in conjunction with the sequence alignments in Fig. 6.1. For this purpose, equivalent residue numbers with respect to the ovine IL-1 proproteins are given in the main text. It is not certain exactly how this structure interacts spatially with either IL-1 receptor although in the orientation shown in Fig. 6.2.a, the majority of highlighted residues are pointing away from the reader, ie. the face of the molecule which interacts with the receptor is facing into the page. In Fig. 6.2.c the molecule has been rotated round all three axes in such a way as to show that the majority of residues supposedly involved in receptor contact (for binding or initiation of signal) are facing out of the protein surface and that the three groups. (The colour coding of groups of residues in figures 6.2.a and 6.2.c are not the same)

Different orientations of the monomer are shown in Fig. 6.2.a (opposite), and Figs. 6.2.b and 6.2.c on the following page. Fig. 6.2.b is tilted slightly but otherwise Figs. 6.2.b and 6.2.c show similar orientations.

Figure 6.2.a

Red	- Group 1:	residues forming a discontinuous IL-1β/IL-1RI binding site (Lys-93 is common to groups 1 and 2)
Dark yellow	- Group 2:	residues involved in nuclear localisation of IL-1β (because of crowding, only 4 of the 7 residues within this cluster have been labelled)
Green/ pale yellow	- Group 3:	residues involved in IL-1α or IL-1β activity rather than receptor binding. (Arg-11 and Asp-12[pale yellow], represent actual IL-1β residues and the rest, are the IL-1β equivalents of IL-1α residues for which no co-ordinates were available).

Residues marked with a white asterisk are different in the human and ovine sequences as shown below. The equivalent ovine residue numbers with respect to Figure 6.1 are given in brackets (see text for details).

Group No.	Residue No. on Figure	Human/Murine IL-1β	Ovine IL-1β
1 (Red)	4	Arg	Gln (117)
	6	Leu	Val (119)
2 (Yellow)	93	Lys	Arg (206)
1,2 (Red)	94	Lys	Asn (207)
3 (Green)	11	Arg	Gln (124)

a.

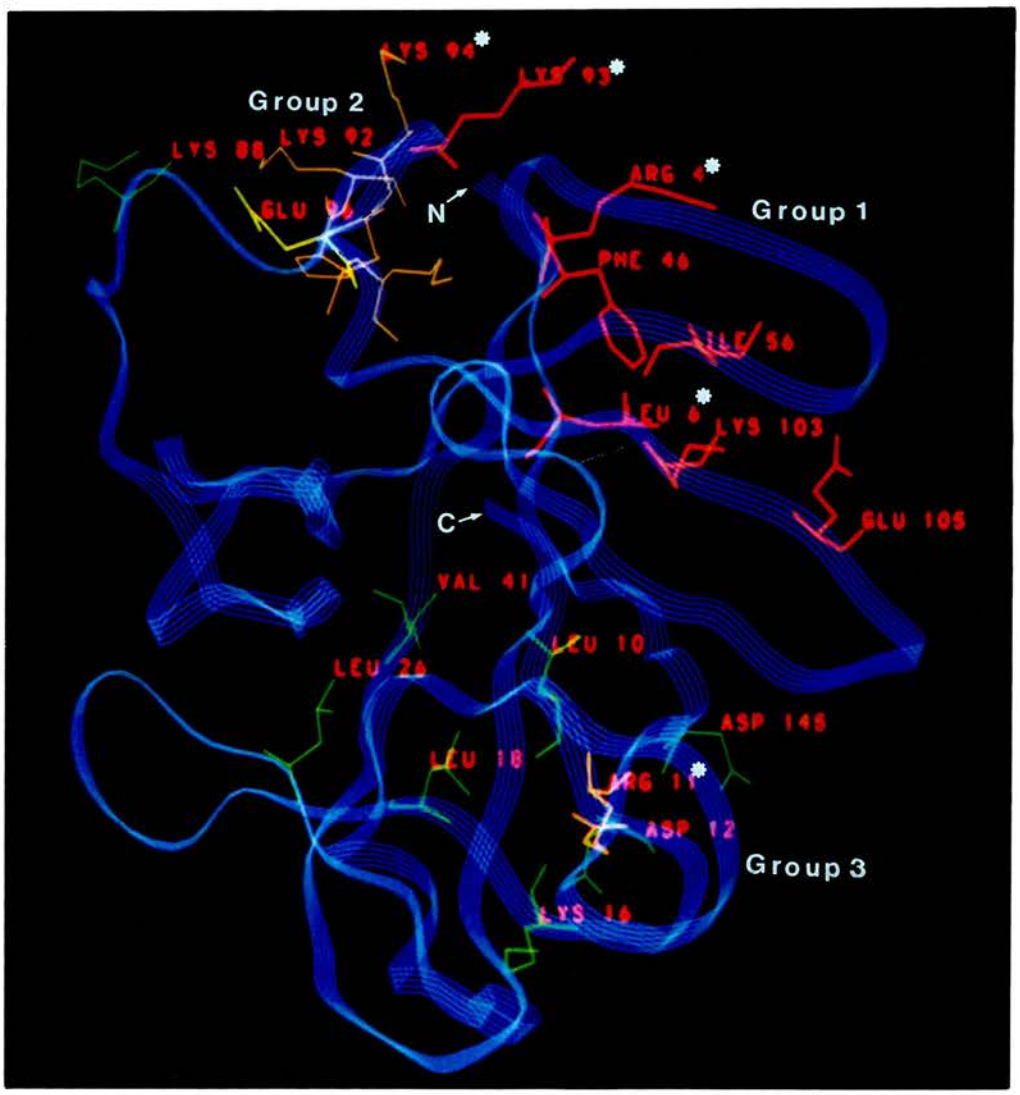

Group 2
LYS 94
LYS 93
LYS 92
LYS 88
GLU 86
N
ARG 4
Group 1
PHE 46
ILE 56
LEU 6
LYS 103
GLU 105
C
VAL 41
LEU 10
LEU 24
ASP 145
LEU 18
ARG 11
ASP 12
Group 3
LYS 14

Figure 6.2.b

Assuming the axes of rotation to be through the centre of the molecule, this figure has been rotated, with respect to Fig. 6.2.a, through about 180° round the x-axis and clockwise a few degrees round the y-axis. Groups 1-3 residues have not been highlighted but their relative positions are indicated for orientation purposes.

Green - residues conserved across IL-1α and IL-1β.
Gold - residues conserved across human IL-1ra and ovine/bovine IL-1β but no other IL-1β sequences. Equivalent ovine proprotein numbers for these 4 residues are given below:

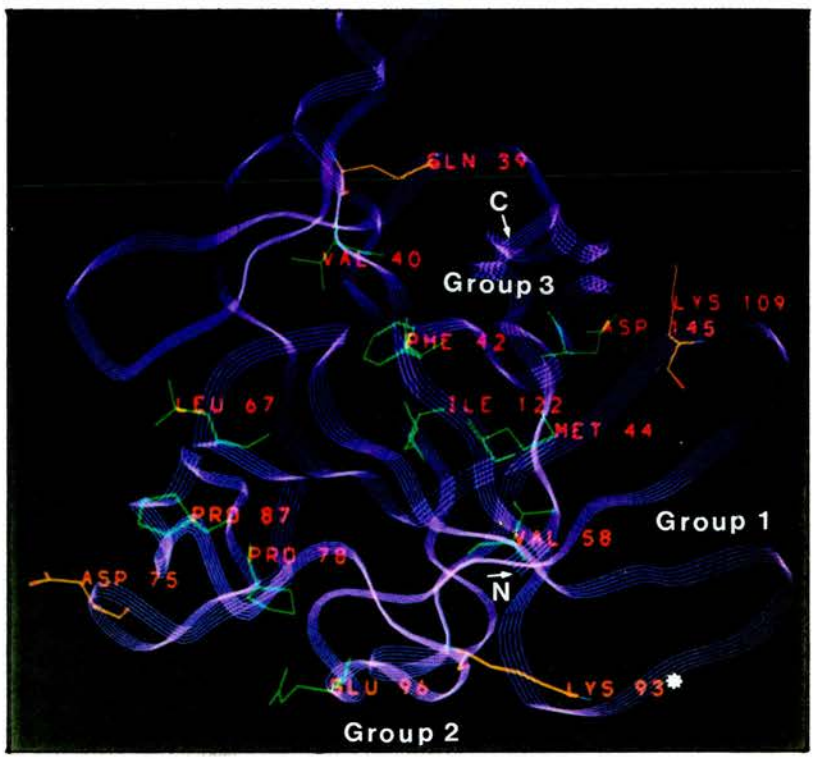
Residue No. on Figure	Human IL-1β	Ovine IL-1β/Human IL-1ra
39	Gln	Glu (152)
75	Asp	Gly (188)
93	Lys	Arg (206)
109	Lys	Tyr (222)

Figure 6.2.c

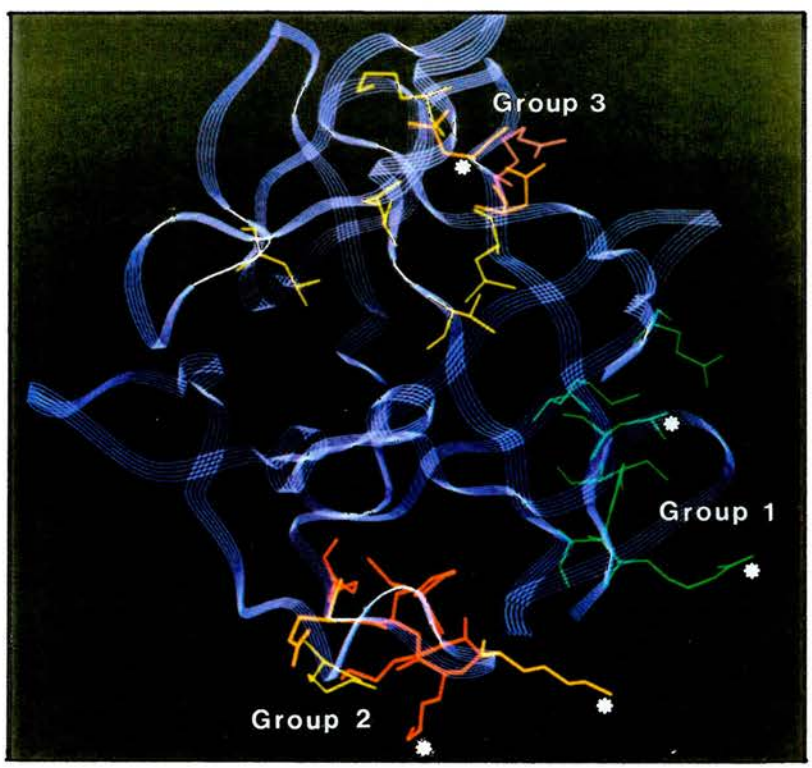
Similar orientation to Fig. 6.2.b but with some further rotation round all axes. Individual residues have not been named but the three main receptor-contact groups are indicated and residues which are different in ovine IL-1β are again indicated with an asterisk.

Green	Group 1:	red in Fig. 6.2.a
Orange/dark yellow	Group 2:	yellow in Fig. 6.2.a
pink/pale yellow	Group 3:	green/pale yellow in Fig. 6.2.a

b.


GLN 39
C
VAL 40
Group 3
LYS 109
ASP 145
PHE 42
LEU 67
ILE 122
MET 44
PRO 87
Group 1
VAL 58
PRO 78
ASP 75
N
GLU 96
LYS 93
Group 2

c.


Group 3
Group 1
Group 2

6.1.2.1 Group 1: IL-1 Residues Important for Binding to Type I IL-1R

It has been proposed that seven residues within human IL-1β form a discontinuous IL-1 type I receptor binding site (Labriola-Tompkins et al., 1991). These residues, which are shown in red on Fig. 6.2.a (and green in Fig. 6.2.c), and are indicated by ▲ in Fig. 6.1, are clustered in one area of the molecule. Of these seven residues, four are fully conserved across all IL-1β sequences, ie. Phe-46(β-159), Ile-56(β-169), Lys-103(β-216) and Glu-105(β-218). Human residues Leu-6(ovine β-Val-119) and Lys-93(ovine β-Arg-206) both show conservative substitutions in the ovine sequence. The seventh residue, Arg-4(β-117), is conserved across all but the ovine and bovine sequences which both contain Gln-117. In addition to structural differences, the pIs of these two amino acids are very different, Arg being pI=10.76 as opposed to Gln, pI=5.65. This amino acid difference could explain in part the reduced biological activity of ovine and bovine IL-1β when assayed on murine cells. In support of this contention, Labriola-Tompkins et al., (1991) showed that mutation of this residue to Asp abrogated binding to both type I and type II receptors indicating that an acidic side chain may not be tolerated at this position. In addition, they showed that even the conservative Lys/Arg-4(117) substitution abrogated receptor binding, which could have implications with respect to other basic/basic amino acid substitutions. Substitution of β-Lys-93 with amino acids containing aromatic, polar or negatively charged side chains all abrogate receptor binding. This residue is conserved across all species except ovine and bovine IL-1β which both have the related β-Arg-206. In this context it may be of interest that the human IL-1 receptor antagonist also has Arg at the equivalent position. Taken as a group therefore, the ovine/human differences at positions β-4(117), β-6(119) and β-93(206) which are in close proximity spatially, as seen in Fig. 6.2, could be a major factor contributing to the species specificity observed in this study.

Only three of these seven IL-1β residues have conserved IL-1α counterparts, β-Arg-4(ov. α-Arg-128), which has been shown to be important for IL-1α/IL-1RI and IL-1RII interactions (Nanduri et al., 1991; Labriola-Tompkins et al., 1991) and β-Leu-6(ov. α-Ile-130) and β-Ile-56(ov. α-Leu-180) both of which are only partially conserved. For IL-1β binding, an aromatic residue at position β-46(159) is essential for receptor binding but all IL-1α sequences have Ala at this position. These residues demonstrate potentially different type I receptor binding requirements for IL-1α and IL-1β.

6.1.2.2 Residues Important for Binding to Type II IL-1R

Few residues have yet been implicated in IL-1β binding to the type II IL-1R. β-Asp-145(β-258) which is important for IL-1RII binding is conserved in the ovine sequence as is Phe-46(β-159). This latter residue and Arg-4(117) are the only two

residues within the proposed IL-1RI discontinuous binding site which have any great effect on IL-1RII binding (Labriola-Tompkins et al., 1991). For both receptor types an aromatic ring is essential at position β-46 and acidic side chains at β-4 reduce binding. Unlike type I requirements, however, type II binding will tolerate mutations to β-Ala-4 or β-Lys-4. Antibody studies have indicated that two sequences, equivalent to ovine residues β-162–165 and β-174–183, are important for human or murine IL-1RII binding but without being directly involved (Boraschi et al., 1991b). These sequences are not highlighted on Fig. 6.2, but can be seen from Fig. 6.1. Amino acids β-162–165 are only partially conserved across species and their importance in this context is unclear, although they are centred within the nona-peptide (β-160-168) which has been advocated for adjuvant use. This latter function, however, involves type I rather than type II receptor generated responses. The second peptide sequence, residues β-174–183, is reasonably well conserved and seven of the amino acids are present in the ovine sequence with the other three being conservative substitutions. Ovine IL-1 binding to macrophages (IL-1RII), from other species was not examined in the study being reported here and the importance of any of these residues with respect to ovine IL-1RII generated bioactivity is unknown.

Residues essential for type II receptor mediated IL-1α activity are not known.

6.1.2.3 Group 2: Residues Involved in Nuclear Localisation of IL-1β

IL-1β bound to the type I receptor is thought to be internalised and translocated to the nuclear membrane either on its own or attached to the receptor. In EL4 cells which possess the type I receptor, the kinetics of nuclear membrane and plasma membrane binding have been shown to be similar (Grenfell et al., 1989). There is nevertheless still controversy as to the significance of IL-1β nuclear binding in the generation of biological responses, as has been detailed in Chapter 1.11.1.4.

Residues within the putatitive IL-1β nuclear localisation sequence, 91-97(β-204-210) are shown in dark yellow on Fig. 6.2.a (orange/dark yellow in Fig. 6.2.b) and form a group distinct from the surface receptor binding group. The putative nuclear localisation sequence present in human, murine, rat and rabbit IL-1β, 91PK**KK**MEK97, is altered to 204PK**RN**MEK210 in both ovine and bovine IL-1β. These alterations appear to be relatively minor, apart from a charge change due to the K/N substitution, and may not restrict nuclear membrane binding. Lys-93(β-206), the only one of these residues common to both plasma membrane and nuclear binding, is situated at the junction between the two groups. The level of conservation of these Group 2 residues strongly suggests functional importance

and Lys-93(206) appears to be a key residue. It may be significant with respect to both human/murine IL-1β activity that hIL-1ra, which binds to the receptor but would not be expected to be internalised, has residues equivalent to β-206 and β-207 but does not contain the full nuclear localisation sequence. That the antagonist has the equivalent of ovine β-Arg-206, not Lys as in the human sequence, may also be relevant in the context of low ovine IL-1β activity on murine cells. IL-1α, which is not thought to be localised to the nucleus, appears to have deletions within this region but does have an LKKRRL motif, bases α-82–87, which might represent a nuclear localisation signal (Grenfell et al., 1988).

Of the residues supposedly involved in surface receptor and nuclear IL-1β binding, all except Arg-4(ovine β-Gln-117), Leu-6(ovine β-Val-119), Lys-93(ovine β-Arg-206) and Lys-94(ovine β-Asn-207) are conserved across all species. From the above analyses, three points in particular are interesting: (i) ovine IL-1β and hIL-1ra both have Arg rather than Lys at position β-206; (ii) there is charge difference in the ovine protein at position β-207; (iii) residues β-4(117) and β-93(206) are in close proximity regardless of orientation of the molecule; all of which may indicate a significant contribution to species specificity by these residues.

6.1.2.4 Group 3: Residues Involved in IL-1 Activity

Mutational studies and chemical modification have so far implicated numerous residues in IL-1 activity but many of the alterations have produced relatively minor effects. The most important residues, as demonstrated by point mutations (Poindexter et al., 1991; Kawashima et al., 1992; Yanofsky et al., 1990; Nanduri et al., 1991; Nakii et al., 1990; Gehrke et al., 1990; Conca et al., 1991), are highlighted on Fig. 6.2.a. All are situated within the common β-sheet motifs. IL-1β residues are pale yellow and IL-1α-equivalent residues are green with numbers being those of the corresponding IL-1β residues (pink and yellow respectively in Fig.6.2.b). IL-1α numbers will therefore be given in brackets to identify the specific residues. It can be seen that these amino acids are spatially separated from those involved in receptor binding and except for Lys-88(α-212, β-201) form a distinct, though somewhat more disperse, grouping. This latter residue is conserved across IL-1α and IL-1β sequences, except for the rabbit which has conservative substitutions, and the equivalent IL-1ra residue is the non-basic Asp, observations which suggest an important role for Lys-88(α-212). Mutational studies have in fact shown that the requirement at this position, for IL-1α at least, is a basic amino acid. Mutation of α-Lys-88 to Arg had no effect on stimulation of A375 cell proliferation whereas mutation to Ile resulted in a 40-60% loss of this activity (Kawashima et al., 1992).

Of the other important IL-1α residues, all are conserved in ovine IL-1α. Only two are not also conserved in ovine IL-1β, Lys-16(α-Gln-142; β-Lys-129) and Val-41(α-Lys166; β-Val-154) and in each case the equivalent IL-1ra residue is Lys. Asp-26(α-138) and Leu-41(α-153) residues are also conserved in IL-1ra. This degree of conservation and the inwardly facing position of Leu-18(α-144) suggests a structural role for these latter three residues. Asp-151(α-262) is conserved in IL-1α and IL-1β but not IL-1ra. Mutation of α-Asp-151 to Tyr resulted in no change in IL-1RI binding affinity but some activities were reduced (Yamaoshi et al., 1990). Its equivalent β-Asp-145(β-258) has been shown to be essential for IL-1β activity generated from the type II receptor but its importance with respect to the type I receptor has not been established.

The remaining amino acid in this group, β-Arg-11(β-124), is β-Gln-124 in both ovine and bovine sequences but is not conserved in IL-1α. Mutation of human Arg-11(β-124) and Arg-4(β-117) reduces T cell activation by lowering receptor binding affinity (Nakai et al., 1990; Gehrke et al., 1990; Conca et al., 1991; Labriola-Tompkins et al., 1991; Chang et al., 1992), and the substitution of β-Gln-124 for Arg at this position could therefore be expected to contribute to the comparatively low ovine IL-1β (this study) and bovine (Maliszewski et al., 1988) activities detected with murine cells. Ovine IL-1α was also less active on murine cells but none of the above residues are obviously involved in this species specificity.

Three residues, Arg-4(α-128; β-117), Asp-12(α-138; β-125) and Asp-145(α-262; β-258) have been shown to be important for selected activities of both IL-1α and IL-1β. Within the Group 3 residues, α-Gln-142 and α-Lys-166 appear to be important for IL-1α activity exclusively and Arg-11(β-124) for IL-1β activity exclusively. Otherwise the conservation within this group might predict that IL-1α and IL-1β illicit signal transduction from IL-1RI via similar residues. Within the human IL-1β C-terminal regions 148-162 and 173-204, which have been shown to be involved in activity but not receptor binding (Boraschi et al., 1991a; Herzberg et al., 1989), eleven residues are conserved across IL-1α and IL-1β in all species. Taken together, these observations could indicate that it is the receptor binding requirements which determine IL-1 activity and that once bound, the IL-1 residues required for initiating signal transduction pathways may not be all that different for IL-1α and IL-1β. Cross-species data however contradicts this view to some extent. Much more data on IL-1/IL-1RI interactions is required before the precise involvement of particular residues can be stated with any certainty.

One further consideration which has to be borne in mind when assessing these residues is that mutation may not necessarily show which residues are being implicated in any particular function. For example, it appears that different residues may be essential for generation of selected activities. Mutation of α-Glu-94 to Val reduces LAF activity by 46% without affecting either A375 cell proliferation or prostaglandin synthesis whereas mutation to Gly does not inhibit any of these functions (Kawashima et al., 1992). However, α-Glu-94 is also conserved in the antagonist. It is equally possible that conformational change induced by mutation of α-Glu-94 alters receptor contact by other residues and that α-Glu-94 is not intrinsically important for initiation of signal transduction.

In the current study, FXa unexpectedly truncated the C-terminal of IL-1βm. Unlike IL-1α, a FXa site is only present in the ovine and bovine sequences. IL-1βm and its truncated form exhibited similar biological activities therefore, if the assumption that IL-1βm is cleaved at β-244 is correct, this would indicate that amino acids β-244-266 may not be absolutely essential for ovine IL-1β activity via the type I receptor and that loss of these amino acids may not adversely affect IL-1β tertiary structure with respect to the ovine receptor requirements. This is in contrast to data showing that deletion of β-253-266 results in loss of activity and that mutation of Lys to Asp in the IL-1ra at the position equivalent to β-Asp-258 results in the antagonist displaying minimal agonist activity (Ju et al., 1991). β-Asp-145(258) is supposedly essential for human IL-1β binding to the type II receptor (Kawashima et al., 1992). In this study, binding of an ^{125}I-IL-1β preparation which contained 50% FXa truncated IL-1β to macrophages, was not significantly different to that of preparations containing only the full length protein (data not shown). This is the only indication that IL-1/IL-1RII interactions may also be different in the sheep.

6.1.2.5 Residues Essential for Maintaining the Structural Integrity of IL-1

Labriola-Tompkins et al., (1991) have shown that β-Met-157 and β-Val-171 are essential for maintaining the structural integrity of IL-1 with respect to binding to either receptor type. These two residues are fully conserved across IL-1α and IL-1β sequences but it is of interest that the equivalent residues in IL-1ra are the related Val and Ala respectively which may mean that the receptor antagonist is slightly more flexible, allowing for less restricted receptor binding.

6.1.2.6 Thoughts on the Requirements for Ovine IL-1/IL-1 Interactions

Two obvious factors are important for ligand/receptor interactions. Firstly that the ligand structure assumes optimal configuration for binding and secondly that within this configuration, individual residues can make appropriate contact for

initiation of the signal transduction cascade. Receptor binding requirements are difficult to assess because so much of the data available in the literature is based on mutational studies which, by definition, must induce some conformational change in the molecule. It is by no means certain that altered activities can be attributed purely to the amino acid(s) mutated. Equally, data derived from antibody studies is dependent on specific epitopes being recognised and results from different studies are not always in agreement. Without more knowledge about potentially important residues, it is difficult to predict exact requirements for activity. That having been said, it appears from the above analyses, that the binding requirements for the sheep IL-1 receptors may be slightly different to those of the human or murine IL-1 receptors. IL-1β residues Gln-117, Gln-124, Arg-206 and Asn-207 all seem to be important with respect to generation of different levels of biological responses from the ovine vs. murine IL-1 type I receptor. No conclusions could be drawn about residues contributing to the similar species specificity exhibited by ovine rIL-1α but α-Val-170 may be a candidate. Residues thought to be required for activity via the IL-1 type II receptor are partially conserved in the ovine protein and this receptor would also be expected to exhibit species specificity although this has not been tested.

All those residues which the above data would suggest are functionally important, are identical in all published ovine and bovine IL-1 sequences with the exception of bovine β-Ile-119 which represents a conservative Val/Ile substitution (Fiskerstrand et al., 1990, 1992; Seow et al., 1991; Andrews et al., 1991; Leong et al., 1988; Maliszewski et al., 1988). In view of the comparatively low level of conservation between human/murine and ovine IL-1 it is likely that the respective tertiary structures might assume slightly different configurations which could implicate different residues in receptor contact and generation of biological responses.

6.2 Biological Activity of the IL-1β Proprotein

Ovine rIL-1β proprotein was found to be 5 times less active than the mature protein in thymocyte assays. By comparison, the human recombinant proprotein is 120 times less active than the mature protein in the D10.G4.1 assay (Jobling et al., 1988). Both these cell types display the type I receptor. Human IL-1βp and IL-1βm separated by chromatofocusing (isoelectric points 5 vs 7 respectively), could also both be bound to EL4 cells (IL-1RI), the binding affinity of the proprotein being 3.5% that of the mature protein (K_d IL-1βm=1x10^{-9}M). In contrast, the soluble IL-1R, a proteolytically cleaved portion of the IL-1 type II receptor, binds the IL-1β proprotein efficiently (Symons et al., 1991b), which may indicate that human and murine IL-1βp function at least, is largely directed

through IL-1RII or an IL-1RII-like receptor. If this were the case, it could explain, in part, the low IL-1β proprotein activities generally reported from T cell or chondrocyte assays. Different folding patterns of IL-1βp and IL-1βm could determine the relative binding affinities for the two cellular receptors or alternatively, the structure of the proprotein may limit its activity and it may be more active if membrane bound.

Whether the activity of pro–β is of any importance remains to be determined but it is interesting to speculate on its possible function. The idea that the IL-1β proprotein may have some restricted function(s) *in vivo,* is attractive from a number of points of view:

(i) Membrane bound forms of IL-1 have been described which are involved in direct cell-cell communication between macrophages and T cells (Nagelkerken and van Breda Vriesman, 1986; Bhardwaj et al., 1989). The main constituent of membrane bound IL-1 seems to be proIL-1α, but the presence of IL-1β has been suggested (Jobling et al., 1988). Platelets express active IL-1α and IL-1β which are entirely membrane bound and rapid delivery of IL-1 to vascular endothelial cells following injury has implicated platelets as providers of an initiating signal for the inflammatory response (Hawrylowicz et al., 1989a, 1991). These membrane IL-1s have not been characterised but may prove to be the proproteins. Membrane bound IL-1 and soluble IL-1 may act via different mechanisms or receptors (Kurt-Jones et al., 1986; Hurme, 1987).

(ii) Keratinocytes constitutively produce large amounts of proIL-1β, only some of which is processed. Cultured keratinocytes do not process the IL-1β at all (Bigler et al., 1992). The specific monocyte IL-1β processing enzyme, ICE, has not been detected in keratinocytes although serine proteases are known to process the proIL-1β to unusual peptides (Cooper et al., 1990a; Hammerberg et al., 1990). It is likely that there could be some as yet unknown, but specific, function for the IL-1β proprotein in these cells. Involvement in localised inflammatory reactions is possible. As I was in the process of writing this chapter, two papers were published, one showing that LPS or IL-1β stimulated human foetal microglial cells produce abundant levels of potent IL-1β which remains primarily cell associated (Lee et al., 1993) and the other that there is reduced secretion of IL-1β by peritoneal cells during continuous ambulatory peritoneal dialysis (Hart et al., 1993). Both suggest a pro-inflammatory role for the cell-associated IL-1β proprotein. That this form of IL-1β may be involved in antigen presentation has also been suggested.

(iii) As many as thirty residues in the N-terminal region of the proproteins are conserved across the IL-1β and a further eleven across both IL-1α and IL-1β. which suggests some functional significance. It is possible that this region contains recognition sites for chaperonin proteins responsible for transport of IL-1β across the cell membrane.

6.3 Genomic Organisation of Ovine IL-1

Human, murine, rabbit and bovine IL-1 genes appear to exist as single copies. Although I did not investigate ovine IL-1 at a genomic level, recently published data from Andrews et al. (1992) suggests that the same is true for the ovine genes. For future study of IL-1 it would be useful to have knowledge of the promoter and regulatory regions within the ovine IL-1 genes. All that is currently known is that the 3' regions of IL-1α and IL-1β cDNAs in the sheep are both AU rich and, in common with other species, contain a number of copies of AUUA motifs thought to be responsible for the short, <30min, mRNA half-lives (Turner et al., 1989; Andrews et al., 1991).

6.4 Production of Ovine IL-1 by LPS Stimulated Alveolar Macrophages

Northern blot analysis of LPS stimulated ovine alveolar macrophages showed production of IL-1α and IL-1β mRNAs to peak around 4-6h (Fig. 3.18) but no data is yet available on the synthesis and secretion of the IL-1 proteins. In other species synthesis of IL-1α is delayed and it appears to exert its influence in a predominantly cell associated form. IL-1β is more rapidly translated and has been thought to require secretion and processing for optimal biological activity (see Chapter 1, Sect. 1.4). It would be of interest to assess the translation rates and export of ovine IL-1, not only by macrophages but also other cell types such as fibroblasts, dendritic cells and platelets. Secretion of IL-1 into the macrophage supernatants could not be determined in this study because TNF, and to a lesser extent IL-6, also act as proliferative agents in the thymocyte and cartilage degradation assays which were used for estimating recombinant IL-1 activities. Routinely employed biological assays specific for human and murine IL-1, such as induction of proliferation of D10.G4.1 or EL4.NOB1 cells, are of no use for measurement of ovine IL-1 activity because of the low species cross-reactivity.

Preliminary work involving non-reducing PAGE gel electrophoresis of LPS stimulated macrophage supernatants has shown that the polyclonal rabbit antiserum raised against ovine-IL-1βm detects a single band on Western blots (data not shown). This band is not detected in unstimulated macrophages but

increases in intensity over the first four hours of LPS stimulation. The band is seen at an apparent molecular weight much higher than expected for either form of IL-1β, ie. at ≥68kDa, which may mean that the IL-1β is either aggregated or bound to a chaperonin. Elucidation of the patterns of ovine IL-1 production awaits the development monoclonal antibodies and specific ovine IL-1 assays.

6.5 IL-1 and Afferent Lymph Dendritic Cells

That numerous cytokines are involved in the induction of antigenic or inflammatory responses is very clear and the extremely complex response to antigen is obviously under very tight cytokine control.

The mechanism whereby IL-1 enhances DC function has not yet been elucidated, neither is it known to what extent IL-1 is involved in the early response to antigen. It is now known that even in non-inflamed skin, LC adhere strongly to keratinocytes via E-cadherin, the expression of which decreases as the LC differentiate into DC (Tang et al., 1993). Cell-associated or membrane-bound keratinocyte IL-1 could therefore potentially act on LC by cell:cell contact.

IL-1R expressed by ovine LC have yet to be assessed but in the current study 300-400 IL-1 receptors were detected on resting DC found in peripheral afferent lymph. Secondary ovalbumin (OVA) skin-challenge, dramatically increased both the number of IL-1α receptors and the proportion of DC expressing IL-1R. Changes in IL-1α binding were much larger than IL-1β and were co-incident with cell numbers; a transient increase by 4h, followed by a second increase to maximum cell output on days 2 and 3 after which the count gradually returned to baseline by day 8. IL-1β binding, in contrast, was increased on days 2-3 but then dropped dramatically.

Antigen uptake by sheep cells after secondary challenge is known to be rapid. Following the injection of 200μg OVA, ≤40% of DC arriving at the lymph node by 40min were positive for FITC-labelled antigen and the response had disappeared by 2h (Harkiss et al., 1990). A larger OVA dose (2mg) resulted in a greater response (70% DC positive for antigen) of longer duration (4h). Having only injected 50μg OVA in the current experiments, a lesser response would be expected but it is conceivable that the individual DC from the 4h lymph collection which bind IL-1α could also be antigen-associated. If IL-1 action is transient, receptors may be internalised rapidly, in which case only a small proportion would be detected. Even after secondary challenge only very few DC showed extremely high IL-1α binding and no more than 10% of DC expressed

detectable IL-1R. IL-1β at all times was bound to a much lesser extent than IL-1α.

Only a very small proportion of non-specific esterase (NSE) positive dendritic cells in afferent lymph were also positive for acetylcholinesterase (AChe). Veiled afferent lymph DC were not strongly positive with either of these stains which, if these veiled cells are most mature form of DC found in afferent lymph, may indicate diminishing lysosomal activity with maturation of the DC. Veiled DC also do not seem to be the cells expressing IL-1 receptors although this can not be stated with absolute certainty from the data obtained. NSE/^{125}IL-1 positive cells appear to be dendritic as do the AChe positive cells. Staining labelled cells with AChe prior to exposure would clarify whether the AChe positive subpopulation of cells are also expressing IL-1R.

The size of the IL-1 receptor operative on ovine DC has not been determined but it seems to exhibit IL-1R type I rather than type II characteristics. This is also claimed to be the case for cultured murine LC/DC (Kampgen et al., 1992). B cell lines exhibit a heterogeneous population of receptors, expressing 68kDa and 80kDa receptors, the latter of which bind IL-1α with a greater affinity than IL-1β (Benjamin et al., 1990). IL-1 can enhance PMN and monocyte FcR mediated phagocytosis (Simms et al., 1991) and the action of IL-1 on PMN could possibly be via the same 80kDa receptor, in which case a parallel might be active in LC/DC. Amplification of receptor cDNA by *in situ* PCR using primers appropriate for each IL-1 receptor type would establish which is expressed by LC/DC lineage cells.

Earlier studies on sheep have shown that MHC class II expression by afferent lymph dendritic cells increased uniformly to almost sixfold by days 4 and 5 following secondary antigen challenge and had returned to resting levels by day 8 (Hopkins et al., 1988). Maximal IL-1R expression precedes increased MHC class II expression by 1-2 days, which may indicate either no correlation or else an indirect association between IL-1 action and class II-restricted antigen presentation. IL-1 does not upregulate Ia on the mature DC surface (Koide et al., 1987a) but a trace population of murine thymic Ia$^-$ DC precursors can be induced to form fully functional Ia$^+$ DC by the action of IL-1 (Inaba et al., 1988). More direct evidence in favour of a connection between IL-1 and class II expression comes from data showing that intracutaneous injection of IL-1β into mice resulted in decreased density of Ia$^+$ DC over 2-7days with the remaining DC showing increased Ia$^+$ expression over days 1-4 (Lundqvist and Back, 1990).

Resting DC express the sheep analogue of human CD1 uniformly. Increased expression of CD1 is also seen from days 2-7 after challenge but only on about 35% of the cells (Bujdoso et al., 1990; Hopkins et al., 1989). CD1 restriction has recently been demonstrated as being potentially important in antigen presentation by peripheral blood DC suggesting a functional parallel between CD1 and MHC class II-restricted responses (Porcelli et al., 1992). CD1a, which is the first marker to appear which distinguishes cultured monocytes from developing macrophages, is specifically expressed by Langerhans cells and thymocytes (van de Rijn et al., 1984). GM-CSF is involved in the generation of DC from LC and has been shown, together with IL-4, to induce CD1a,-b,-c expression on blood monocytes (Porcelli et al., 1992). $CD1a^+$, $CD1b^+$ and $CD1c^+$ afferent lymph DC have all been detected in sheep. The role of CD1 in the antigen presentation by lymph DC is as yet not understood, and the spectrum of cytokine influence is unknown. The involvement of IL-4 in a secondary response may prove to be closely linked to that of IL-1. In addition to its ability to upregulate CD1, IL-4 has also been shown to decrease human FcγR expression (Chin et al., 1990; te Velde et al., 1992; Bieber, 1993) which would correlate with the observed FcR involvement in antigen uptake followed by decreased FcR expression prior to antigen presentation in sheep (Harkiss et al., 1990). More importantly, IL-4 can upregulate IL-1RI expression (Koch et al., 1992).

In view of the temporal co-incidence of the very early IL-1R expression and antigen uptake, and the later IL-1R and CD1 expression, it would interesting to determine the relationship between IL-1R expressing DC, OVA-associated DC and DC expressing CD1 subclasses. If a clear relationship does exist and if the IL-1 receptors are concentrated over the region of juxtanuclear vesicles, as might be indicated by the pattern of ^{125}I-IL1 grains on the exceptionally hot cells, this data, together with evidence that IL-1 enhances antigen presentation through its action on the DC, could point to a role for IL-1α with respect to antigen uptake or processing. In support of an early role for IL-1α, intravenous injection of rIL-1α into mice has shown a concommitant rapid cell depletion of spleen and thymus which may represent mobilisation of immunocompetent cells during the development of the immune inflammatory response. Very few DC are required to present antigen and a ratio of 1:200 DC:TC is sufficient to induce a strong response in *in vitro* assays (Klinkert and Steffen, 1988). In culture, IL-1β mRNA transcription is upregulated during DC maturation (Heufler et al., 1992) and during *in vitro* antigen presentation to T cells, DC/T cell clusters form which induce IL-1α mRNA synthesis but no IL-1 has yet been detected in cell supernatants. Cell-associated IL-1α and/or IL-1β could have a role in antigen

presentation, the DC IL-1 acting on T cells via cell:cell contact. Evidence for this was suggested as early as 1986 by the finding that DC from rat thoracic duct lymph possess membrane-associated structures identical to or mimicing IL-1 which are involved in T cell activation (Nagelkerken and van Breda Vriesman, 1986) and more recently evidence has been put forward that membrane associated IL-1α may play a role in activating antigen-presenting cells, albeit peripheral blood monocytes and B-lymphocytes, in the initiation of immune responses (Eugui and Almquist, 1990).

Keratinocytes constitutively produce mRNAs for a number of cytokines without actively secreting the cytokine, IL-1 being a prime example. Of the other cytokines, IL-10 is secreted in response to contact allergens reaching peak activity by 12h (Enk and Katz, 1992) and although IL-10 has been implicated along with secretion of TNFα and IL-1ra in the suppression of DTH responses after UV irradiation, a differential mechanism of release may exist whereby positive and negative effects of the cytokines are regulated (Rivas). IL-10 is also capable of inducing FcR expression while suppressing MHC class II expression in macrophages (Chin et al., 1991; te Velde et al., 1992).

6.5.1 Speculation about the Role of IL-1 in Secondary Responses to Localised Ovalbumin Challenge

From what is currently known in the sheep, a sequence of events following secondary ovalbumin challenge could be envisaged which include putative IL-1 actions: i) antigen is taken up by DC, possibly via FcR; ii) keratinocyte derived IL-1α and possibly keratinocyte and/or LC derived IL-1β could be involved in initiating mobilisation of LC and maturation into DC; iii) CD1 and IL-1R expression are increased while FcR expression is down regulated; iv) IL-1α might indirectly influence antigen processing in the late endocytic vesicles; v) MHC class II becomes upregulated; vi) processed antigen is presented to specific T cell subsets via MHC class II and/or CD1 ligands.

The importance of supplementary cytokines including IL-4, IL-6, IL-8, IL-10, TGFβ, IFNγ and GM-CSF with respect to positive and negative regulation of intermediate stages between antigen administration and antigen presentation to T cells has yet to be determined in the sheep.

An enhancing role in antigen presentation has previously been ascribed to IL-1. The results of this study do not dispute this and it has been confirmed that IL-1, and in particular IL-1α, does act directly on dendritic cells. LC/DC IL-1β mRNA is massively upregulated in culture where DC:T cell contact is not an

issue (Heufler et al., 1992). It is tempting to speculate that IL-1α or some other cytokine might induce transcription of IL-1β mRNA but that the signal for translation comes from DC:T cell contact and IL-1β acts in an entirely cell associated form during clustering. IL-1α may also be induced and would also act in a membrane associated form. Such a scheme could explain why the enhancing effects of IL-1 appear to be directed purely at DC not T cells and why anti-IL-1 can not inhibit this enhancement. There are precedents for disconnected actions of IL-1α and IL-1β. For example, Boraschi et al. (1990) demonstrated that IL-1α and IL-1β might perform different functions with respect to immune responses to sheep red blood cells. Both forms were involved equally in inflammatory reactions but only IL-1β was immunostimulatory and it appeared that IL-1α could act as a negative regulator of this immunostimulation.

6.5.2 Projections

LC/DC derived IL-1β has been shown to be involved in T cell priming responses after initial antigen challenge (Enk et al., 1993) but it appears to play a lesser role in secondary responses (Ruco et al., 1990). Could the view that during secondary responses IL-1α is involved in LC/DC mobilisation and antigen processing whereas IL-1β is primarily involved in antigen presentation and T cell reactions, be correct? Much still needs to be established, such as; (i) exactly at which stage IL-1 is exerting its effect; (ii) whether IL-1α and IL-1β are serving different functions in the early response to secondary antigen challenge; (iii) whether IL-1 synthesis is induced during the response and if so, at what point and is IL-1 or some other stimulus responsible; (iv) whether IL-1α could induce IL-1β mRNA transcription and the signal for translation be supplied by T cell contact; (v) whether the enhancing effect of IL-1 on antigen presentation may purely be due to upregulation of adhesion molecules.

Information on the regulation of DC function could also become of interest in wider contexts eg. in various cancers, in which infiltrates of DC have been found which are thought to participate in anti-tumour reactions (Peng, 1991, Tsujitani et al., 1992) and in the development of autoimmune diseases such as diabetes (Tafuri et al., 1993).

Future work may need to take into account the regulatory effects of both IL-1ra and sIL-1R. Keratinocyte IL-1ra remains intracellular which would support a role for localised control of keratinocyte IL-1. With differentiation of the keratinocytes, the IL-1ra/IL-1 ratio increases, showing increased IL-1ra mRNA transcription (Bigler et al., 1992; Gruaz-Chatellard et al., 1991). This is also true for both monocyte and keratinocyte types of IL-1ra in psoriatic skin

(Hammerberg et al., 1992). Non-secreted IL-1ra may therefore serve to regulate autocrine IL-1-mediated pathways of growth/differentiation. In addition, although dexamethasone does not affect LC production of IL-1, glucocorticoid treatment of epidermal cells abolishes IL-1 but not IL-1ra production (Stosic-Grujicic and Lukic, 1992) indicating that immunosuppressive effects of glucocorticoids as well as inflammatory effects of IL-1α in human skin can be modified by IL-1ra. sIL-1R, on the other hand, appears to be predominantly immunosuppressive, indicating a major role for IL-1β in the induction of immune responses.

The expression of IL-1 by LC or DC in the sheep either in response to cytokines including IL-1, or as a result of antigen challenge, has not yet been determined. Probes available as a result of this project could be used for detection of IL-1 mRNA by *in situ* hybridisation or *in situ* PCR and IL-1 mRNA transcription could be followed throughout a secondary response. Standard PCR detection methods would not be a viable alternative in this instance because of the sensitivity of PCR and the extreme difficulty in obtaining pure cell preparations. Although IL-1 production by ovine keratinocytes has also not yet been studied, these cells would be expected to produce cell-associated IL-1α and IL-1β, thus providing an abundant source of IL-1 in the skin.

6.6 Conclusions

In conclusion, the main aims of this project have been met. The ovine IL-1 cDNAs have been cloned and biologically active recombinant ovine IL-1 proteins have been expressed. The latter are being used to raise monoclonal and polyclonal antibodies.

Experiments using radiolabelled recombinant ovine IL-1α and IL-1β have established that ovine afferent lymph dendritic cells express IL-1 receptors which are upregulated during a secondary immune response to ovalbumin challenge.

The cloned cDNAs, recombinant proteins and antisera will all be of use in further elucidating the role of IL-1 in various pathological conditions in the sheep.

APPENDIX 1

Summary of IL-1 Receptor Expression on Various Cell Types

This table has been compiled from published data in order to give an idea of the variations in receptor characteristics which have been detected by different groups. The species from which the cells or tissue have been derived is indicated and the IL-1 species used for receptor detection is given if different. Cells possessing the type I IL-1R are listed first. The size of the receptor and percentage of cells expressing detectable receptors has been inserted if known, as has data on the effect of upregulating or inhibitory agents on IL-1 receptor expression. In some cases only the size of the receptor has been determined. Data on both high and low affinity sites is included and related figures are bracketed }. Although this list is reasonably comprehensive, it by no means covers all reported studies.

Cell Type	IL-1	IL-1R M_r kDa	Treatment (if any)	R/cell	% +ve cells	K_d(pM)	Reference
Cells possessing IL-1R type I:							
T cell:							
EL4-6.1$_m$	α	80/120	-	4000	-	55	Savage et al., 1989
	α	nd	-	400	-	5	Lowenthal et al., 1986
				19600		500	"
	α_h	87	nt	6700-14200	-	1000	Bomsztyk et al., 1989a,b
			PMA	6200	-	1000	"
	α_h	80	-	2800	-	625	Dower et al., 1986a
	β_n	80	-	2400	-	560	"
	α	80	nt	22656	-	1000	Scapigliati et al., 1989
			DEX	"	-	"	"
	β	80	nt	2988	-	360	"
			DEX	"	-	"	"
	βp	80/100	-	nd	-	nd	Bird et al., 1987
	α/β	nd	-	241	-	400	Horuk et al., 1987
	α/β	nd	-	1565	-	200-360	Bird et al., 1988a,b
	α/β	100	-	850-1857	-	20-87	Chizzonite et al., 1989a
D10.G4.1$_m$	α/β	nd		1256	-	107	Chizzonite et al., 1989a
D10S$_m$	α	73/46	-	3300-23800	-	227	Savage et al., 1989
Thymocyt$_m$	α	73/46	PHA	44	-	270	Takeuchi et al., 1992
				230		2500	"
L3T4^+_m	α	nd	nt	9	-	2-4	Lowenthal et al., 1987
				230-800		175-430	
			PMA	27	-	2-4	"
				850		175-430	
YT-C3$_h$	α	80	-	600	-	40	Bensimon et al., 1989
				7000		7000	"
LBRM-33-IA5$_m$	α_h	80	-	3430	-	217	Dower et al., 1986
	β_h	80	-	3200	-	3333	"
	β_n	80	-	2800	-	74	"
	β	nd	-		-	300-1400	"
NK		70	-	nd	-	nd	Bensimon et al., 1989
T lympho		78	-	nd	-	nd	Thieme et al., 1989
PB T_h	α/β	nd	nt	40	-	300	Shirakawa et al., 1987
			ConA	350	-	300	"
Epithelial:							
NMuLi$_m$	α/β	nd	-	790	-	70	Chizzonite et al., 1989a

Cell Type	IL-1	IL-1R M_r kDa	Treatment (if any)	R/cell	% +ve cells	K_d(pM)	Reference
Keratinocyte:							
h	α/β	nd	nt	300-600	-	200-400	Blanton et al.,1989
			PMA	3400	-		"
h	α/β	72	-	nd	-	nd	Kupper et al., 1988
PAM-212$_m$	α/$\beta_{h,m}$	72	-	1900	-	52	Chizzonite et al., 1989a
Chondrocyte:							
p	α	nd	-	7000	-	250	Saklatvala et al., 1986
r	β	nd	nt	1620	-	0.1	Chandrasekhar et al., 1989
			FGF	2960	-	0.1	"
h	α/β	nd	-	3000-5000	-	nd	McCollum et al., 1991
			IL-1	1000	-	nd	"
h	α/β	nd	-	2315	-	30	Martel-Pelletier et al., 1992
OA$_h$	α/β	nd	-	4069	-	30	"
Fibroblast:							
Synovial$_p$	α/β	97	-	3-5000	-	160	Bird et al., 1986
		80	-	nd	-	nd	Dower et al., 1985
Synov-Rheum$_h$	α	nd	-	3000-14000	90-100	66	Chin et al., 1988
	β	nd	-	3000-14000	-	4	"
MLg-2908$_m$	α/$\beta_{h,m}$	nd	-	2900	-	32	Chizzonite et al., 1989a
lung$_m$	α/$\beta_{h,m}$	nd	-	2.1fmol/mg	-	23	"
lung$_h$	β	80/200	-	3000	10-15	8.4	Chin et al., 1987
TIG-1$_h$	α	80	nt	26000	-	510	Takii et al., 1992
			PGE_2	38000	-	360	"
		nd	IL-1		-	770	Pronost et al., 1993
h	α	78	-	nd	-	nd	Thieme et al., 1989
dermal$_h$	α	nd	nt	1600	-		Akahoshi et al., 1988b
			PGE_2	5400	-		"
	α	80	-	2800	-	625	Dower et al., 1986a
	β_n	80	-	2400	-	560	"
dermal$_m$	α/β	80	-	3-5000	-	100	Bird and Saklatvala, 1986
dermal$_b$	β	72	-	130	-	49	Lederer and Czuprynski 1993
				2500		3700	
diploid$_h$	α/β	nd	-	5000-15000	-	63	Qwarnstrom et al., 1988
diploid$_m$	α/β	nd	-	2900-5673	-	32-57	Chizzonite et al., 1989a
	α/β	nd	PDGF	up 4-5x	-		Bonin and Singh, 1988
	α/β	74	-	nd	-	nd	Bird et al., 1985
BALB-c/3T3$_m$	α_h	78	-	5500	-	333	Dower et al., 1986a
	β_n	78	-	4800	-	476	"
	β_p	80/100	-	nd	-	nd	Bird et al., 1987
3T3.Li$_m$	α/$\beta_{h,m}$	nd	-	220-570	-	10-60	Chizzonite et al., 1989a
3T3.Swiss$_m$	α/$\beta_{h,m}$	nd	-	5673	-	57	"
Osteoblast:							
m	α/β_p	nd	-	3000-5000	-	100	Bird and Saklatvala, 1986
MC3T3.E1	α/β	100	-	6500	-	102	Shelly and Laborde, 1992
Endothelial:							
h	α	78	-	100-500	-	~90	Thieme et al., 1989
s.END.1$_m$	α	80	-	1273	-	95	Boraschi et al., 1991c
t.END.1$_m$	α	80	-	771	-	85	"
Smooth muscle:							
vascular$_h$	α	78	-	nd	-	nd	Boraschi et al., 1991c

Cell Type	IL-1	IL-1R M_r kDa	Treatment (if any)	R/cell	% +ve cells	K_d(pM)	Reference
Brain:							
hypothal-	β	?I	-	75fmol/mg	-	1300	Katsuura et al., 1988
cortex	β	?I	-	17fmol/mg	-	1300	"
pituitary	β	?I	-	<<	-	nd	"
pit- AtT-20_m	α	nd	-	1500	-	150	Bristulf et al., 1991
Kidney:							
$cortex_m$	α	nd	nt	34.7fmol/mg	-	6.6	Takao et al., 1991
			LPS	11.3fmol/mg	-	6.6	"
$medulla_m$	α	nd	nt	52.7fmol/mg	-	6.6	"
			LPS	26.0fmol/mg	-	6.6	"
Cells possessing IL-1R type II:							
Bone marrow:							
m	$\alpha/\beta_{h,m}$	nd	-	233-549	-	244-294	Chizzonite et al., 1989a
Macrophage:							
$J774A.1_m$	$\alpha/\beta_{h,m}$	80	-	387-1196	-	133-347	Chizzonite et al., 1989a
$P\ 388\ D1_m$	$\alpha/\beta_{h,m}$	80	-	604	-	59	"
$AJM_2.C11_m$	$\alpha/\beta_{h,m}$	80	-	1755	-	348	"
Monocyte:							
h	β	80	-	100	-	600	Uhl et al., 1989
THP-1	α	64	nt	0	-	-	Spiggs et al., 1990
	"		DEX	2000	-	300	"
	β	64	nt	0	-	-	"
	"		PMA	0	-	-	"
	"		PGE_2	0	-	-	"
	"		DEX	0	-	-	"
	"		PMA +PGE_2	1000	-	2000	"
	"		PMA + DEX	4000	-	"	"
	"		PMA + PGE_2 +DEX	32000	-	"	"
PMN:							
h	α	nd	-	700	-	2800	Rhyne et al.,1988
h	α/β	nd	-	900	-	300	Parker et al., 1989
m	α/β	nd	-	1700	-	300	"
b	β	71	-	-	-	-	Lederer and Czuprynski, 1992
B cell:							
B_h	α	nd	nt	100	-	260	Akahoshi et al., 1988a
			GC	2000	-	260	"
	α	68/80	-	14-866	-	100-300	Benjamin et al., 1990
		"	-	238-15817	-	5200-16000	"
	β	"	-	253-8171	-	1500-7700	"
MC116	α	"	-	67	-	120	"
	β	"	-	43	-	430	"
B_h	α/β	"	nt	70	3-5	4	Tanaka et al., 1989
				320	3-5	380	
			SAC	30	20	4	"
				1960	20	380	
$RAJI_h$	α	68	nt	316	-	2400	Scapigliati et al., 1989
			DEX	incr.	-	2400	"
	β	nd	nt	2400	-	130	"
			DEX	incr.	-	130	"
	β	nd	PMA	decr.	-	nd	"

Cell Type	IL-1	IL-1R M_r kDa	Treatment (if any)	R/cell	% +ve cells	K_d(pM)	Reference
$RAJI_{h(cont.)}$	β	68	nt	1941	5-16	2100	Stoppacciaro et al., 1991
			DEX	9205	14-30	1100	"
	β	nd	-	nd	-	2200	Symons et al., 1991
	α/β	68	-	7709	-	2100	Horuk et al., 1989
$1H7_h$	β	67	-	15524	15	700	Stoppaciaro et al., 1991
			DEX	48000	28	1100	"
			PMA	<10	0.5	nd	"
$STS\ 25_h$	β	71	nt	3174	16	800	"
			DEX	13467	29	1300	"
Daudi	α/β	?	nt	<10	0	nd	Horuk et al., 1989 /
			DEX	<10	0	nd	Stoppaciarro et al., 1991 / Benjamin et al., 1990
$3B6_h$	α	70	-	300	-	60	Bensimon et al., 1989
				6000		5000	
$ARH77_h$	α	?	-	<<	-	nd	Dower et al., 1986
	β	nd	-	11000	-	480	"
	β	nd	-	1100	-	2000	"
	β_n	nd	-	11300	-	2273	"
PreB:							
$70Z/3_m$	α_h	66	nt	100	-	60	Bomsztyk et al., 1989a,b
				900		140	
			PMA	76	-	53	"
				166		1400	
		60	-	nd	-	nd	Matsushima et al., 1989a
	$\alpha/\beta_{h,m}$	82	-	616-930	-	244-522	Chizzonite et al., 1989a
Langerhans' cell:							
BALB-cm	α/β	80	-	>500	-	<100	Kampgen et al., 1992
C3H/Hem	α/β	80	-	>500	-	<100	"
Cultured Dendritic cell:							
BALB-cm	α/β	80	-	100	-	<100	Kampgen et al., 1992
C3H/Hem	α/β	80	-	100	-	<100	"
Pancreas:							
β-cells	α	?	-	0	-	nd	Hammonds et al., 1990
	β	nd	-		-	200	"
						1400	"
Carcinoma:							
MDA-MB-415	β	nd	-	700	-	880	Gaffney et al., 1988
$HBL\text{-}100_h$	α/β	"	-	<<	-	nd	"
colon $SW48_h$	α/β	"	-	700	-	900	"
$melanoma_h$	β	"	-	100-1000	-	160	Bauman et al., 1993
" A375-C6	α	"	nt	701	-	19	Usui et al., 1991
" A375-C5	α	"	nt	58	-	17	"
			IL-1α	?	-	19	"
			IL-1α	?	-	17	"
RAJI:	β	47 (sIL-1R)		na		2700	Symons et al., 1991

h - human; m - mouse; p - pig; r - rabbit; n - purified natural IL-1
GC - glucocorticoids; synov-RA - rheumatoid synoviocytes; PB - peripheral blood; OA - osteoarthritic
sIL-1R - soluble IL-1R.
For other abbreviations see pp. xv-xvii
nt - no treatment; nd - not determined; na - not applicable; ? - not known or not stated
} - high and low affinity sites from Scatchard plots

APPENDIX 2

Examples of Cytokine Networking

The effects of cytokines on cytokine expression can vary depending on the target cell (see Chapter 1, Sect. 1.5), for example, IL-4 stimulates IL-1 expression in B cells but inhibits IL-1, IL-6 and TNFα expression in macrophages. The cytokine (Cyto.) effects listed below apply principally to monocytes and macrophages, being the prime controllers of cytokine networking.
Cytokine effects on MHC class II expression by macrophages have been included because of potential relevance in the context of antigen presentation.

1st Cyto.	2nd Cyto.	Stimulated	Inhibited	No effect
IL-1	-	LIF,GM-CSF,G-CSF		
"	-	IL-1,IL-2,IL-6,IL-8,		M-CSF
"	-	PGE_2/cAMP		
"	IFNγ		IL-1	
"	LPS		TNFα	
"	TGFβ		IL-6	
"	TNFα	IL-6		
IL-4	-		IL-1,IL-6,TNFα	
IL-6	-		IL-1,TNFα	
IL-10	IFNγ		IL-1,IL-6,IL-8,TNFα,	
"	"		GM-CSF	
"	"		MHC Class II	
TNFα		IL-1,IL-6,LIF,M-CSF		
"	IFNγ	IL-1,		
IFNγ	-	Class II		IL-1,TNFα
"	GM-CSF	IL-1,TNFα		
"	"	Class II, HLA-DR		
GM-CSF	-	IL-1,TNFα, HLA-DR		
TGFβ	-	LIF	IL-1,TNFα	IL-6

For abbreviations see pp. xv-xvii

REFERENCES

Abbott F, Tam FW, Ryan JJ, Rees AJ (1992) Human mesangial cells synthesise interleukin 1 alpha but not interleukin 1 beta , interleukin 1 receptor antagonist or tumour necrosis factor. *Nephrology, Dialysis, Transplantation* **7**:997-1001

Abernethy NJ, Hay JB, Kimpton WG, Washington E, Cahill RNP (1991) Lymphocyte subset-specific and tissue-specific lymphocyte-endothelial cell recognition mechanisms independently direct the recirculation of lymphocytes from blood to lymph in sheep. *Immunology* **72**:239-245

Adams SE, Dawson KM, Gull, Kingsman SM, Kingsman AJ (1987a) The expression of hybrid HIV:Ty virus like particles in yeast. *Nature* **329**:68-70

Adams SE, Mellor J, Gull K, Sim RB, Tuite MF, Kingsman SM, Kingsman AJ (1987b) The functions and relationships of Ty-VLP proteins in yeast reflect those of mammalian retroviral proteins. *Cell* **49**:111-119

Adams SE, Richardson MH, Kingsman SM, Kingsman AJ (1991) Expression vectors for the construction of hybrid Ty-VLPs. In: M Collins (Ed.), *Methods in molecular biology,* vol**8**: (viral vector and transfection techniques). Humana Press Inc., Clifton, NJ, Chapter 24.

Akahoshi T, Oppenheim JJ, Matsushima K (1988a) Induction of high-affinity interleukin-1 receptor on human peripheral blood lymphocytes by glucocorticoid hormones. *J Exp Med* **167**:924-936

Akahoshi T, Oppenheim JJ, Matsushima K (1988b) Interleukin-1 stimulates its own receptor expression on human fibroblasts through the endogenous production of prostaglandin(s). *J Clin Invest* **82**:1219-1224

Akira S, Isshiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y, Nakajima T, Hirano T, Kishimoto T (1990) A nuclear factor for IL-6 expression (NF-IL-6) is a member of a C/EBP family. *EMBO J* **9**:1897-1906

Andrews AE, Barcham GJ, Brandon MR, Nash AD (1991) Molecular cloning and characterisation of ovine IL-1α and IL-1β. *Immunology* **74**:453-460

Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rahmsdorf HJ, Jonat C, Herrlich P, Karin M (1987) Phorbol ester-inducible genes contain a common cis element recognised by a TPA-modulated trans-acting factor. *Cell* **49**:729-739

Aotsuka S, Nakamura K , Nakano T, Kawakami M, Goto M, Okawa-Takatsuji M, Kinoshita M, Yokohari R (1991) Production of intracellular and extracellular interleukin-1 alpha and interleukin-1 beta by peripheral blood monocytes from patients with connective diseases. *Ann Rheum Dis* **50**:27-31

Arend WP, Joslin FG, Massoni RJ (1985) Effects of immune complexes on production by human monocytes of interleukin-1 or an interleukin-1 inhibitor. *J Immunol* **134**;3868-3875

Arend WP, Joslin FG, Thompson RC, Hannum CH (1989) An IL-1 inhibitor from human monocytes. Production and characterisation of biologic properties. *J Immunol* **143**:1851-1858

Arend WP, Welgus HG, Thompson RC, Eisenberg SP (1990) Biological properties of recombinant human monocyte-derived interleukin-1 receptor antagonist. *J Clin Invest* **85**:1694-1697

Arend WP, Coll BP (1991) Interaction of recombinant monocyte-derived interleukin-1 receptor antagonist with rheumatoid synovial cells. *Cytokine* **5**:407-413

Arend WP, Smith MF, Janson RW, Joslin FG (1991b) IL-1 receptor antagonist and IL-1 beta production in human monocytes are regulated differently. *J Immunol* **147**:1530-1536

Arend WP (1991a) Interleukin 1 receptor antagonist. A new member of the interleukin 1 family. *J Clin Invest* **88**:1445-1451

Arkema JM, Schadee-Eestermans IL, Broekhuis-Fluitsma DM, Hoefsmit EC (1991) Localisation of class II molecules in storage vesicles, endosomes and lysosomes in human dendritic cells. *Immunobiology* **183**:396-407

Atkins E (1960) Pathogenesis of fever. *Physiol Rev* **40**:580-646

Auron PE, Webb AC, Rosenwasser LJ, Mucci SF, Rich A, Wolff SM, Dinarello CA (1984) Nucleotide sequence of human monocyte interleukin-1 precursor cDNA. *Immunology* **81**:7907-791

Aznar C, Fitting C, Cavaillon JM (1990) Lipopolysaccharide-induced production of cytokines by bone marrow-derived macrophages: dissociation between intracellular interleukin 1 production and interleukin 1 release. *Cytokine* **2**:259-265

Bailly S, Ferrua B, Fay M, Gougerot-Pocidalo MA (1990) Differential regulation of IL-6, IL-1 A, IL-1 B and TNF alpha production in LPS-stimulated human monocytes: role of cyclic AMP. *Cytokine* **2**:205-210

Baker SM, Johnston SA, Hopper JE, Jeahning JA (1987) Transcription of multiple copies of the yeast GAL7 gene is limited by specific factors in addition to GAL4. *Mol Gen Genet* **208**:127-134

Bakouche O, Brown DC, Lachman LB (1987) Subcellular localisation of human monocyte interleukin 1: evidence for an inactive precursor molecule and a possible mechanism for IL-1 release. *J Immunol* **138**:4249-4255

Baldari C, Murray JAH, Ghiara P, Cesarini G, Galeotti CL (1987) A novel leader peptide which allows efficient secretion of a fragment of human interleukin 1β in *Saccahromyces cerevisiae*. *EMBO J* **6**:229-234

Baldari CT, Telford JL (1989) The intracellular precursor of IL-1 beta is associated with microtubules in activated U937 cells. *J Immunol* **142**:785-791

Ballou LR, Chao CP, Holness MA, Barker SC, Raghow R (1992) Interleukin-1 mediated PGE2 production and sphyngomyelin metabolism. Evidence for the regulation of cyclooxygenase gene expression by sphingosine and ceramide. *J Biol Chem* **267**:20044-20050

Bandara G, Lin CW, Georgescu HI, Evans CH (1992) The synovial activation of chondrocytes: evidence for complex cytokine interactions involving a possible new factor. *Biochim Biophys Acta* **1134**:309-318

Banks WA, Ortiz L, Plotkin SR, Kastin AJ (1991) Human Interleukin (IL) 1α and murine IL-1β are transported from blood to brain in the mouse by a shared saturable mechanism. *J Pharmacol Exp Therapeut* **259**:988-996

Barak V, Perritt D, Flechner I, Yanai P, Halpern T, Treves AJ, Dinarello CA (1991) The specific IL-1 inhibitor from the human M20 cell line is distinct from the IL-1 receptor antagonist. *Lympho Cytokine Res* **10**:437-442

Barkley DE, Feldman M, Maini RN (1990) Cells with dendritic morphology and bright interleukin-1 alpha staining circulate in the blood of patients with rheumatoid arthritis. *Clin Exp Immunol* **80**:25-31

Bartolini G, Orlandi M, Chiricolo M, Licastro F, Zambonelli P,Minghetti L, Tomasi V (1990) Interleukins and interferons: yin-yang modulators of PGH synthetase in human macrophages. *Biofactors* **2**:267-270

Bayliss MT (1990) Proteoglycan structure and metabolism during maturation and ageing of human articular cartilage. *Biochem Soc Trans* **18**:799-802

Beck G, Habicht GS (1986) Isolation and characterisation of a primitive interleukin-1-like protein from an invertebrate, *Asterias forbesi*. *Proc Natl Acad Sci USA* **83**:7429-7433

Beekhuizen H, Corsel-van Tilburg AJ, Blokland I, van Furth R (1991) Characterisation of the adherence of human monocytes to cytokine-stimulated human macrovascular endothelial cells. *Immunology* **74**:661-669

Beelen RHJ, Steenbergen JJE, van Vugt E, Betjes MHG, Havenith CEG, Kamperdijk EWA (1992) Dendritic cells isolated rom rat and human non-lymphoid tissue are very potent accessory cells. *2nd Int symposium on dendritic cells in fundamental and clinical immunology, Amsterdam, The Netherlands. Abstracts*, p25

Beeson PB (1948) Temperature-elevating effect of a substance obtained from polymorphonuclear leukocytes. *J Clin Invest* **27**:524

Bell J (1989) The polymerase chain reaction. *Immunol Today* **10**:351-355

Bendtzen K, Mandrup-Poulsen T, Nerup J, Nielsen JH, Dinarello CA, Svenson M (1986) Cytotoxicity of human pI interleukin-1 for pancreatic Islets of Langerhans. *Science* **232**:1545-1547

Bendtzen K, Svenson M, Jonsson V, Hippe E (1990) Autoantibodies to cytokines - friends or foes? *Immunology Today* **11**:167-169

Benjamin D, Wormsley S, Dower SK (1990) Heterogeneity in interleukin-1 receptors expressed on human B cell lines. *J Biol Chem* **265**:9943-9951

Bensi G, Raugei G, Palla E, Carinci V, Tornese BD, Melli M (1987) Human interleukin-1 beta gene. *Gene* **52**:95-101

Bensimon C, Wakasugi H, Wakasugi N, Jitsuwaka S, Tursz T (1989) A monoclonal antibody recognising 68- to 75-kilodalton protein associated with the human IL-1 receptor. *J Immunol* **142**:2290-2298

Berger AE, Carter DB, Hankey SO, McEwan RN (1993) Cytokine regulation of the interleukin-1 receptor antagonist protein in U937 cells. *Eur J Immunol* **23**:39-45

Berman MA, Sandborg CI, Calabia BS, Andrews BS, Friou GJ (1987) Interleukin 1 inhibitor masks high interleukin-1 production in acquired immunodeficiency syndrome (AIDS). *Clin Immunol Immunopathol* **42**:133-140

Bernaudin JF, Yamauchi K, Wewers MD, Tocci MJ, Ferrans VJ, Crystal RG (1988) Demonstration by in situ hybridisation of dissimilar IL-1 beta gene expression in human alveolar macrophages and blood monocytes in response to lipopolysaccharide. *J Immunol* **140**:3822-3829

Bertoglio JH (1988) B-cell-derived human interleukin 1. *Crit Rev Immunol* **8**:299-313

Besedovsky H, Rey AD, Sorkin E, Dinarello CA (1986) Immunoregulatory feedback between interleukin-1 and glucocorticoid hormones. *Science* **233**:652-654

Betjes MG, Haks MC, Tuk CW, Beelen RH (1991) Monoclonal antibody EBM11 (anti-CD68) discriminates between dendritic cells and macrophages after short-term culture. *Immunobiology* **183**:79-87

Beuscher H, Gunther C, Rollinghof M (1991) IL-1 beta is secreted by activated murine macrophages as biologically inactive precursor. *J Immunol* **144**:2179-2183

Beuscher HR, Fallon RJ, Colten HR (1987) Macrophage membrane interleukin 1 regulates the expression of acute phase proteins in human hepatoma Hep 3B cells. *J Immunol* **139**:1896- 1901

Beuscher HU, Nickells MW, Colten HR (1988) The precursor of interleukin-1 alpha is phosphorylated at residue serine 90. *J Biol Chem (HIV)* **263**:4023-4028

Bhardwaj N, Lau LL, Rivelis M, Steinman RM (1988) Interleukin-1 production by mononuclear cells from rheumatoid synovial effusions. *Cell Immunol* **114**:405-423

Bhardwaj N, Lau LL, Friedman SM, Crow MK, Steinman RM (1989) Interleukin 1 production during accessory cell-dependent mitogenesis of T lymphocytes. *J Exp Med* **169**:1121-1136

Bieber T (1993) FceRII/CD23 on epidermal Langerhans' cells. *Res Immunol* **143**:445-447

Bigler CF, Norris DA, Weston WL, Arend WP (1992) Interleukin-1 receptor antagonist production by human keratinocytes. *J Invest Dermatol* **98**:38-44

Binns RM, Licence ST, Wooding FB, Duffus WP (1992) Active lymphocyte traffic induced in the periphery by cytokines and phytohemagglutinin: three different mechanisms? *Eur J Immunol* **22**:2195-2203

Birbeck BS, Breathnach AS, Everall JD (1961) An electron microscope study of basal melanocytes and high level clear cells (Langerhans cells) in vitiligo. *J Invest Dermatol* **37**:51-64

Bird TA, Saklatvala J (1986) Identification of a common class of high affinity receptors for both types of porcine interleukin-1 on connective tissue cells. *Nature* **324**:263-266

Bird TA, Gearing AJ, Saklatvala J (1987) Murine interleukin-1 differences in binding properties between fibroblastic and thymoma cells and evidence for a two-chain model. *FEBS Lett* **225**:21-26

Bird TA, Gearing AJ, Saklatvala J (1988a) Studies on the murine interleukin-1 receptor. *Dev Biol Stand* **69**:83-91

Bird TA, Gearing AJH, Saklatvala J (1988b) Murine Interleukin 1 Receptor. Direct identification by ligand blotting and purification to homogeneity of an interleukin 1-binding glycoprotein. *J Biol Chem* **163**:12063-12069

Bird TA, Saklatvala J (1990) Down-modulation of EGF receptor affinity in fibroblasts treated with IL-1 or TNF is associated with phosphorylation at a site other than Thr 654. *J Biol Chem* **265**:235-240

Bird TA, Schule HD, Delaney PB, Sims JE, Thoma B, Dower SK (1992) Evidence that MAP (mitogen-activated protein) kinase activation may be a necessary but not sufficient signal for a restricted subset of responses in IL-1-treated epidermoid cells. *Cytokine* **4**:429-440

Black R, Kronheim M, Cantrell M, Deeley C, March C, Prickett K, Wignal J, Conlon P, Cosman D, Hoop T, Mochizuki D (1988) Generation of biologically active interleukin 1β by proteolytic cleavage of the inactive precursor. *J Biol Chem* **263**:9437-9442

Blakemore AIF, Tarlow JK, McDowell, Wilson G, Zhang G, di Giovine F, Duff GW (1992) SSCP analysis of cytokine genes. *Br. Soc. Immunology, Abstracts, Spring meeting, Sheffield University* :41(3.9)

Blanton RA, Kupper TS, McDougall JK, Dower S (1989) Regulation of interleukin-1 and its receptor in human keratinocytes. *Proc Natl Acad Sci* **86**:1273-1277

Bloom BR, Salgame P, Diamond B (1992) Revisiting and revising suppressor T cells. *Immunology Today* **13**:131-136

Bomsztyk K, Stanton TH, Smith LL, Rachie NA, Dower SK (1989a) Properties of interleukin-1 and interferon-gamma receptors in B thmphoid cell line. *J Biol Chem* **264**:6052-6057

Bomsztyk K, Sims JE, Stanton TH, Slack J, McMahan CJ, Valentine MA, Dower SK (1989b) Evidence for different interleukin-1 receptors in murine B- and T-cell lines. *Proc Natl Acad Sci USA* **86**;8034-8038

Bomsztyk K, Toviola B, Emery DW, Rooney JW, Dower SK, Rachie NA, Sibley CH (1990) Role of cAMP in interleukin-1 induced k light gene expression in murine B cell line. *J Biol Chem* **265**:9413-9417

Bonin PD, Singh JP (1988) Modulation of interleukin 1 receptor expression and interleukin 1 response in fibroblasts by platelet-derived growth factor. *J Biol Chem* **263**:11052-11055

Boraschi D, Villa L, Volpini G, Boss'u P, Censini S, Ghiara P, Scapligiati G, Nencioni L, Bartalini M, Matteucci G (1990a) Differential activity of interleukin 1 alpha and interleukin 1 beta in the stimulation of the imune response *in vivo*. *Eur J Immunol* **20**:317-321

Boraschi D, Antoni G, Perin F, Villa L, Nencioni L, Ghiara P, Presentini R, Tagliabue A (1990b) Defining the structural requirements of a biologically active domain of human IL-1β. *Eur Cytokine Network* **1**:21-26

Boraschi D, Villa L, Ghiara P, Presentini R, Bossu P, Censini S, Nucci D, Massone A, Rossi R, Flad HD, et al (1991a) Differential inhibition of IL-1 beta activities and receptor binding by monoclonal antibodies mapping within a discrete region of the protein. *Lymphokine Cytokine Res* **10**:377-184

Boraschi D, Tagliabue A (1991b) Human interleukin 1: structure-function relationships. *Ann Ist Super Sanita* **26**:273-282

Boraschi D, Rambaldi A, Sica A, Ghiara P, Colotta F, Wang JM, de Rossi M, Zoia C, Remuzzi G, Bussolino F, et al (1991c) Endothelial; cells express the interleukin-1 receptor type I. *Blood* **78**:1262-1267

Borth W, Luger TA (1989) Identification of α_2-macroglobulin as a cytokine binding plasma protein. Binding of interleukin-1β to "F" α_2-macroglobulin. *J Biol Chem* **264**:5818-5825

Borth W, Urbanski A, Prohaska R, Susanz M, Luger TA (1990a) Binding of recombinant interleukin-1 beta to the third complement component and alpha 2-macroglobulin after activation of serum by immune complexes. *Blood* **75**:2388-2395

Borth W, Scheer B, Urbansky A, Luger TA, Sottrup-Jensen L (1990b) Binding of IL-1 beta to alpha-macroglobulins and release by thioredoxin. *J Immunol* **145**:3747-3754

Boultwood J, Breckon G, Birch D, Cox R (1989) Chromasomal localization of murine interleukin-1 alpha and beta genes. *Genomics* **5**:481-485

Bowers WE, Ruhoff MS, Goodell EM (1990) Conditioned medium from rat macrophages and the recombinant factors, IL-1β and GM-CSF, enhance the accessory activity of dendritic cells. *Immunobiology* **180**:362-384

Boyce BF, Aufdermorte TB, Garrett IR, Yates AJ, Mundy GR (1989) Effects of interleukin-1 on bone turnover in normal mice. *Endocrinol* **125**:142-150

Bradford MM (1979) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* **72**:248-254

Brakenhoff RH, Schoenmakers JGG, Lubsen NH (1991) Chimeric cDNA clones: a novel PCR artifact. *Nucl Acids Res* **19**:1949

Breel M, Mebuis RE, Kraal G (1987) Dendritic cells of the mose recognised by two monoclonal antibodies. *Eur J Immunol* **17**:1555-1559

Bristulf J, Simoncsits A Bartfai T (1991) Characterisation of a neuronal interleukin-1 receptor and the corresponding mRNA in the mouse anterior pituitary cell line AtT-20. *Neurosci Lett* **128**:173-176

Brody DT, Durum SK (1989) Membrane IL-1: IL-1α precursor binds to the plasma membrane via a lectin-like interaction. *J Immunol* **143**:1183-1187

Broudy VC, Kaushansky K, Harlan JM, Adamson JW (1987) Interleukin 1 stimulates human endothelial cells to produce granulocyte-macrophage colony-stimulating factor and granuloctye colony-stimulating factor. *J Immunol* **139**:464-468

Brown R, Li Z, Vriend CY, Nirula R, Janz L, Falk J, Nance DM, Dyck DG, Greenberg AH (1991) Suppression of splenic macrophage interleukin-1 secretion following intracerebroventricular injection of interleukin-1β: Evicence for pituitary-adrenal and sympathetic control. *Cell Immunol* **132**:84-89

Brynskov J, Hansen MB, Reimert C, Bendtzen K (1991) Inhibitor of interleukin-1 alpha and interleukin-1 beta-induced T-cell activation in serum of patients with active Crohn's disease. *Dig Dis Sci* **36**:737-742

Bucana CD, Munn CG, Song MJ, Dunner K Jr, Kripke ML (1992) Internalisation of Ia molecules into Birbeck Granule-like structures in murine dendritic cells. *J Invest Dermatol* **99**:365-373

Bujdoso R, Hopkins J, Dutia BM, Young P, McConnell I (1989) Characterisation of sheep afferent lymph dendritic cells and their role in antigen cariage. *J Exp Med* **170**:1285-1302

Bujdoso R, Harkiss G, Hopkins J, McConnell I (1990) Afferent lymph dendritic cells: a model for antigen capturc and presentation *in vivo*. *Intern Rev Immunol* **6**:177-186

Buluwela L, Forster A, Bochm T, Rabbitts TH (1989) A rapid procedure for colony screening using nylon filters. *Nucl Acid Res* **17**:452

Bursten SL, Locksley RM, Ryan JL, Lovett DH (1988) Acylation of monocyte and glomerular mesangial cell proteins. Myristyl acylation of the interleukin 1 precursors. *J Clin Invest* **82**:1479-1488

Butkowski RJ, Elion J, Downing MR, Main KG (1977) Primary structure of human prethrombin 2 and α-thrombin. *J Biol Chem* **252**:4942-4957

Calder VL, Prickett TCR, McKenzie JL, Hart DNJ (1992) Analysis of cytokine and cytokine receptor production by human dendritic cells. *2nd Int symposium on dendritic cells in fundamental and clinical immunology, Amsterdam, The Netherlands. Abstracts*, p17

Cameron PM, Limjuco GA, Chin J, Silberstein L, Schmidt JA (1986) Purification to homogeneity and amino acid sequence analysis of two anionic species of human interleukin-1. *J Exp Med* **164**:237-250

Camussi G, Tetta C, Bussolino F, Andres G, Turello E, Baglioni C (1990) Involvement of cytokines and platelet-activating factor in renal pathology. *J Lipid Mediat* **2**Suppl:P S203-213

Canning PC, Neill JD (1989) Isolation and characterisation of interleukin-1 from bovine polymorphonuclear leukocytes. *J Leukocyte Biol* **45**:21-28

Canning PC, Baker PE (1990) Selective alteration of bovine neutrophil responses by bovine interleukin-1 beta. *Vet Immunol Immunopathol* **26**:1-12

Cannon JG, Dinarello CA (1985) Increased plasma interleukin-1 activity in women after ovulation. *Science* **227**:1247-1249

Capper SJ, Kalinka S, Mander TH (1990) Specific radioimmunassays for IL 1 alpha and IL 1 beta in plasma at physiological and acidic pH: determination of immunreactive forms by gel filtration and radioligand binding studies. *Cytokine* **2**:182-189

Carruth LM, Demczuk S, Mizel SB (1991) Involvement of a calpain-like protease in the processing of the murine interleukin 1 alpha precusor. *J Biol Chem* **266**:12162-12167

Carter DB, Deibel AE, Dunn CJ, Tomich CSC, Laborde AL, Slightom JL, Berger AE, Bienowski MJ, Sun FF, McEwan RN et al (1990) Purification and biological characterisation of interleukin-1 receptor antagonist protein. *Nature* **344**:633-637

Casagli MC, Borri MG, Bigio M, Rossi R, Nucci D, Bossu P, Boraschi D, Antoni G (1989) Different conformation of purified human recombinant recombinant interleukin-1 beta from Escherichia coli and Saccharomyces cerevisiae is related to different levels of biological activity. *Biochem Biophys Res Commun* **162**:357-363

Caussy D, Sauder DN (1989) The role of interleukin-1 in the immunological response. *Transfus Med Reviews* **111**:194-205

Caux C, Dezutter-Dambuyant C, Schmitt D, Banchereau J (1992) GM-CSF and TNF-α cooperate in the generation of dendritic Langerhans cells.*Nature* **360**:258-261

Cavaillon JM, Haeffner-Cavaillon N (1990) Signals involved in interleukin-1 synthesis and release by lipopolysaccharide-stimulated monocytes/macrophages. *Cytokine* **2**:313-329

Cerretti DP, Kozlosky CJ, Mosley B, Nelson N, Van Ness K, Greenstreet TA, March CJ, Kronheim SR, Druck T et al (1992) Molecular cloning of the interleukin-1β converting enzyme. *Science* **256**:97-100

Chain BM, Bou Gharios G, Ohlsen I (1989) Endopeptidase activityies associate with the plasma membrane compartment of an antigen-presenting B cell. *Clin Exp Immunol* **75**:87-92

Chandrasekhar S, Harvey AK (1989) Induction of interleukin-1 receptors on chondrocytes by fibroblast growth factor: a possible mechanism for modulation of interleukin-1 activity. *J Cell Physiol* **138**:136-246

Chandrasekhar S, Harvey AK, Higginbotham JD, Horton WE (1990) Interleukin-1-induced suppression of type II collagen gene transcription involves DNA regulatory elements. *Exp Cell Res* **191**:105-114

Chang JY, Ngai PK, Priestle JP, Joss U, Vosbeck KD, van Oostrum J (1992) Identification of a reactive lysyl residue (Lys103) of recombinant human interleukin-1 beta. Mechanism of its reactivity and imoplication of its functional role in receptor binding. *Biochemistry* **31**:2874-2878

Chantry D, Turner M, Brennan F, Kingsbury A, Feldmann M (1990) Granulocyte-macrophage colony stimulating factor induces both HLA DR expression and cytokine production by human monocytes. *Cytokine* **2**:60-67

Chedid M, Shirakawa F, Naylor P, Mizel SB (1989) Signal transduction pathway for IL-1. Involvement of a pertussis toxin sensitive GTP binding protein in the activation of adenyl cyclase. *J Immunol* **142**:4301-4306

Chedid M, Mizel SB (1990) Involvement of cyclic AMP-dependent protein kinases in the signal transduction pathway for interleukin-1. *Mol Cell Biol* **10**:3824-3827

Chedid M, Yoza BK, Brooks JW, Mizel SB (1991) Activation of AP-1 by IL-1 and phorbol esters in T cells. Role of protein kinase A and protein phosphatases. *J Immunol* **147**:867-873

Chensue SW, Davey MP, Remick DG, Kunkel SL (1986) Release of interleukin-1 by peripheral blood mononuclear cells in patients with tuberculosis and active inflammation. *Infect Immun* **52**:341-343

Chensue SW, Shmyr-Forsch C, Weng A, Otterness IG, Kunkel SL (1989) Biologic and immunohistochemical analysis of macrophage interleukin-1 alpha, -1 beta and tumor necrosis factor production during the peritoneal exudative response. *J Leukoc Biol* **46**:529-537

Chensue SW, Warmington KSW, Berger AE, Tracey DE (1992) Immunohistochemical demonstration of interleukin 1 receptor antagonist protein and interleukin 1 in human lymphoid tissue and granulomas. *Am J Pathol* **140**:269-275

Chin J, Cameron PM, Rupp C, Schmidt J (1987) Identification of a high affinity receptor for native human interleukin 1 beta and interleukin 1 alpha on normal human lung fibroblasts. *J Exp Med* **165**:70-86

Chin J, Rupp E, Cameron PM, MacNaul KL, Lotke PA, Tocci MJ, Schmidt JA, Bayne EK (1988) Identification of a high-affinity receptor for interleukin 1 alpha and interleukin 1 beta on cultured rheumatoid synovial cells. *J Clin Invest* **82**:420-426

Chin JE, Winterrowd GE, Krzesicki RF, Sanders ME (1990) Role of cytokines in inflammatory synovitis. The co-ordinate regulation of intercellular adhesion molecule I and HLA class I and class II antigens in rheumatoid synovial fibroblasts. *Arthritis Rheum* **33**:1776-1786

Chin YH, Cai J-P, Xu X-M (1991) Tissue-specific homing receptor mediates lymphocyte adhesion to cytokine-stimulated lymph node high endothelial venule cells. *Immunology* **74**:478-483

Chirgwin JM, Przybyla AE, MacDonald RJ, Ruffer WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294-5299

Chizzonite R, Griffin M, Parker KP, Truitt T, Yodoi J, Kilian PL, Stern AS (1989b)

Chizzonite R, Truitt, Kilian PL, Stern AS, Nunes P, Parker KP, Kaffka KL, Chua AO, Lugg DK, Gubler U (1989a) Two high affinity interleukin 1 receptors represent separate gene products. *Proc Natl Acad Sci USA* **86**:8029-8033

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guadinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**:156-159

Clark BD, Collins KI, Gandy MS, Webb AC, Auron PE (1986) Genomic sequence for human prointerleukin 1 beta: possible evolution from a reverse transcribed prointerleukin 1 alpha gene.(published erratum appears in Nucl Acids Res 1987, **15**:868) *Nucl Acids Res* **14**:7897-7913

Clore GM, Bax A, Driscoll PC, Wingfield PT, Gronenborn AM (1990) Assignment of the side-chain 1H and 13C resonances of interleukin-1 beta using double- and triple-resonance heteronuclear three-dimensional NMR spectroscopy. *Biochemistry* **29**:8171-8184

Clore GM, Gronenborn AM (1991a) Structures of larger proteins in solution: three- and four-dimensional heteronuclear NMR spectroscopy. *Science* **252**:1390-1399

Clore GM, Wingfield PT, Gronenborn AM (1991b) High-resolution three-dimensional structure of interleukin 1 beta in solution by three- and four-dimensional nuclear magnetic resonance spectroscopy. *Biochemistry* **30**:2315-2323

Clouse KA, Powell D, Washington I, Poli G, Strebel K, Farrar W, Barstad P, Kovacs J, Fauci AS, Folks TM (1989) Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. *J Immunol* **142**:431-438

Cohen PJ, Katz SI (1992) Cultured human Langerhans cells process and present intact protein antigens. *J Invest Dermatol* **99**:331-336

Conca W, Auron PE, Aoun-Wathne M, Bennett N, Seckinger P, Welgus HG, Goldring SR, Eisenberg SP, Dayer JM, Krane SM et al (1991) An interleukin 1 beta point mutant demonstrates that jun/fos expression is not sufficient for fibroblast metaloproteinase expression. *J Biol Chem* **266**:16265-16268

Conlon PJ, Grabstein KH, Alpert A, Prickett KS, Hopp TP, Gillis S (1987) Localisation of human mononuclear cell interleukin 1. *J Immunol* **139**:98-xxx

Conti P, Dempsey RA, Reale M, Barbacane RC, Panara MR, Bongrazio M, Mier JW (1991) Activation of human natural killer cells by lipopolysaccharide and generation of interleukin-1 alpha, beta, tumour necrosis factor and interleukin-6. Effect of IL-1 receptor antagonist. *Immunology* **73**:450-456

Cooper KD, Hammerberg C, Baadsgaard O, Elder JT, Chan LS, Taylor RS, Voorhees JJ, Fisher G (1990a) Interleukin -1 in human skin: dysregulation in psoriasis. *J Invest Dermatol* **95**:24S-26S

Cooper KD, Hammerberg C, Baadsgaard O, Elder JT, Chan LS, Saunder DN, Voorhees JJ, Fisher G (1990b). IL-1 activity is reduced in psoriatic skin: decreased IL-1 alpha and increased non-functional IL-1 beta. *J Immunol* **144**:4593-4603

Copeland NG, Silan CM, Kingsley DM, Jenkins NA, Cannizzaro LA, Croce CM, Huebner K, Sims JE (1991) Chromosomal location of murine and human IL-1 receptor genes. *Genomics* **9**:44-50

Corbett JA, Wang JL, Hughes JH, Wolf BA, Sweetland MA, Lancaster JR, McDaniel ML (1992) Nitric oxide and cyclic GMP formation induced by interleukin 1β in Islets of Langerhans. Evidence for an effector role of nitric oxide in islet dysfunction. *Biochem J* **287**:229-235

Cornall RJ, Prins JB, Todd JA, Pressey A, DeLarato NH, Wicker LS, Peterson LB (1991) Type I diabetes in mice is linked to the interleukin-1 receptor and Lsh/Ify/Bcg genes on chromosome 1. *Nature* **353**:262-264

Cotran RS, Pober JS (1990) Cytokine-endothelial interactions in inflammation, immunity and vascular injury. *J Am Soc Nephrol* **1**:225-235

Cozzolino F, Rubartelli A, Aldinucci D, Sitia R, Torcia M, Shaw A (1989) Interleukin 1 as an autocrine growth factor for acute myeloid leukemia cells. *Proc Natl Acad Sci USA* **86**:2369-2373

Crawford RM, Finbloom DS, Ohara J, Paul WE, Meltzer MS (1987) B cell stimulatory factor-1 (interleukin-4) activated macrophages for increased tumoricidal activity and expression of Ia antigens. *J Immunol* **139**:135-141

Cronkhite RI, Lobick JJ, Plate JM (1993) Heterogeneity of type-II interleukin-1 receptors. Heterogeneity of B-cell interleukin-1 binding created by dimerisation of type-II interleukin-1 receptors. *Human Immunol* **36**:128-136

Cumberbatch M, Gould SJ, Peters SW, Kimber I (1991) MHC class II expression by Langerhans' cells and lymph node dendritic cells: possible evidence for maturation of Langerhans' cells following contact sensitisation. *Immunology* **74**:414-419

Cumberbatch M, Kimber I (1992) Dermal tumour necrosis factor-α induces dendritic cell migration to draining lymph nodes, and possibly provides one stimulus for Langerhans' cell migration. *Immunology* **75**:257-263

Cunningham BC, Henner DJ, Wells JA (1990) Engineering human prolactin to bind to the human growth hormone receptor. *Science* **247**:1461-1465

Curran T, Franza BR Jr (1988) Fos and Jun: The AP-1 connection. *Cell* **55**:395-397

Curtis BM, Widmer MB, deRoos P, Qwarnstrom EE (1990) IL-1 and its receptor are translocated to the nucleus. *J Immunol* **144**:1295-1303

Cybulski MI, Colditz IG, Movat HZ (1986) The role of Interleukin-1 in leukocyte emigration induced by endotoxin. *Am J Pathol* **124**:367-372

D'Eustachio P, Jadidi S, Fuhlbrigge RC, Gray PW, Chaplin DD (1987) Interleukin-1 alpha and beta genes: linkage on chromasome 2 in the mouse. *Immunogenetics* **26**:339-343

Dal Nogare AR (1991) Septic shock. *Am J Med Sci* **302**:50-65

Darmady EM, Davenport SGT (1963) Enumeration of leukocytes. *Hematological techniques* Publ. J and A Churchill Ltd., London. p114

Daumy GO, Wilder CL, Merenda JM, McColl AS, Geoghegan KF, Otterness IG (1991) Reduction of biological activity of murine recombinant interleukin-1 beta by selective deamidation at asparagine-149. *FEBS Lett* **278**:98-102

Dayer JM (1991) Chronic inflammatory joint diseases: natural inhibitors of interleukin 1 and tumor necrosis factor alpha. *J Rheumatol Suppl* **27**:71-75

De Bruijn MLH, Nieland JD, Harding CV, Melief CJM (1992) Processing and presentation of intact hen egg-white lysozyme by dendritic cells. *Eur J Immunol* **22**:2347-2352

de Duve C, Wattiaux R, Baudhuin P (1962) Distribution oof enzymes between subcellular fractions in animal tissues. *Adv Enzymol* **24**:291-294

de Waal Malefyt R, Abrams J, Bennett B, Figdor C, de Vries J (1991) IL-10 inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* **174**:1209-1220

DeChiara TM, Young D, Semionow R, Stern AS, Batula-Bernado C, Fielder-Nagy C, Kaffka K, Kilian PL, Yamazaki S, Mizel SB, Lomedico PT (1986) Structure-function analysis of murine interleukin 1: Biologically active peptides are at least 127 amino acids long and are derived from the carboxyl terminus of a 270-amino acid precursor. *Proc Natl Acad Sci USA* **83**:8303-8307

Dejana E, Breviaro F, Erroi A, Bussolino F, Mussoni L, Gramse M, Pintucci G, Casali B, Dinarello CA, Van Damme J, Mantovani A (1987) Modulation of endothelial cell finctions by different molecular species of interleukin 1. *Blood* **69**:695-699

Demarchez M, Asselineau D, Czernielewski J (1993) Migration of Langerhans cells into human epidermis of "reconstructed" skin, normal skin, or healing skin, after grafting onto the nude mouse. *J Invest Dermatol* **100**:648-652

DeMarco D, Kunkel SL, Strieter RM, Basha M, Zurier RB (1991) Interleukin-1 induced gene expression of neutrophil activating protein (interleukin-8) and monocyte chemotactic peptide in human synovial cells. *Biochem Biophys Res Commun* **174**:411-416

Demeter J, Medzihradsxky D, Kha H, Goetzl EJ, Turck CW (1991) Isolation and partial characterisation of the structures of fibroblast activating factor-related proteins from U937 cells. *Immunology* **72**:350-354

Denham S, Barfoot RK (1992) Synthesis of nitric oxide by dendritic cells activated with gamma IFN. *2nd Int symposium on dendritic cells in fundamental and clinical immunology, Amsterdam, The Netherlands. Abstracts*, p46

Dewhirst FE, Stashenko PP, Mole JE, Tsuramachi T (1985) Purification and partial sequence of human osteoclast-activating factor: Identity with interleukin 1 beta. *J Immunol* **135**:2562-2568

di Giovine FS, Takhish E, Sim E, Duff GW (1992) Genetic polymorphism in the human IL-1β promoter. *British Soc. Immunology, Abstracts,Spring meeting, Sheffield University* :42(3.13)

DiBattista JA, Martel-Pelletier J, Wosu LO, Sandor T, Antakly T, Pelletier JP (1991) Glucocorticoid receptor mediated inhibition of interleukin-1 stimulated neutral metalloprotease synthesis in normal human chondrocytes. *J Clin Endocrinol Metab* **72**:316-326

Dimmeler S, Ankarcrona M, Nicotera P, Brune B (1993) Exogenous nitric oxide (NO) generation or IL-1β-induced intracellular NO production stimulates inhibitory auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase in RINm5F cells. *J Immunol* **150**:2964-2971

Dinarello CA, Conti P, Mier JW (1986) Effect of purified interleukin-1 on on natural killer cell activity: is fever a host defence mechanism for tumor killing?*Yale J Biol Med* **59**:97-106

Dinarello CA, Ikejima T, Warner SJ, Orencole SF, Lonneman G, Cannon JG, Libby P (1987) Interleukin-1 induces interleukin-1. I. Induction of circulating interleukin-1 in rabbits in vivo and in human mononuclear cell in vitro. *J Immunol* **139**:1902-1910

Dinarello CA, Wolff SM (1993) The role of interleukin-1 in disease. *New Eng J Med* **328**:106-113

Dinarello CA (1984) Interleukin-1. *Rev Infect Dis* **6**:51-95

Dinarello CA (1991a) Interleukin-1 and interleukin-1 antagonism. *Blood* **77**:1627-1652

Dinarello CA (1991b) Inflammatory cytokines: interleukin-1 and tumor necrosis fector as effector molecules in autoimmune disease. *Curr Opin Immunol* **3**:941-948

Dingle JT, Saklatvala J, Hembry R, Tyler J, Fell HB, Jubb R (1979) A cartilage Catabolic Factor from Synovium. *Biochem J* **184**:177-180

Dofferhof AS, Vellenga E, Limburg PC, van Zanten A, Mulder PO, Weits J (1991) Tumour necrosis factor (cachectin) and other cytokines in septic shock: a review of the literature. *Neth J Med* **39**:45-62

Dower SK, Kronheim CJ, March CJ, Conlon PJ, Hopp TP, Gillis S, Urdal DL (1985) Detection and characterisation of high affinity plasma membrane receptors for human interleukin 1. *J Exp Med* **162**:501-515

Dower SK, Call SM, Gillis S, Urdal DL (1986a) Similarity between the interleukin 1 receptors on a murine T-lymphoma cell line and on a murine fibroblast cell line. *Proc Natl Acad Sci USA* **83**:1060-1065

Dower SK, Kronheim SR, Hopp TP, Cantrell M, Deeley M, Gillis S, Henney CS, Urdal DL (1986b) The cell surface receptors for interleukin-1α and interleukin-1β are identical. *Nature* **324**:266-268

Dower SK, McMahan C, Flack J, Grubin C, Lupton S, Moseley B, Sims GE (1990) Molecular characterisation of two types of interleukin-1 receptor coding peptides on murine and human cells. *J Leukoc Biol Suppl* **1**:103-

Dower SK, Wignall J, Schooley K, McMahan CJ, Jackson JL, Prickett KS, Lupton S, Cosman D, Sims JE (1989) Retention of ligand binding activity by the extracellular domain of the IL-1 receptor. *J Immunol* **142**:4314-4320

Dubois CM, Ruscetti FW, Keller JR, Oppenheim JJ, Hestdal K, Chizzonite R, Neta R (1991) In vivo interelukin-1 (IL-1) administration indirectly promotes type II receptor expression on hematopoetic bone marrow cells: novel mechanism for the hematopoetic effects of IL-1. *Blood* **78**:2841-2847

Dunlap NE, Tilden AB (1991) T helper/inducer ($CD4^+$) cells prestimulated with PPD induce monocytes to produce interleukin-1 beta. *J Leukoc Biol* **49**:542-547

Dutia BM, McConnell I, Ballingall KT, Keating P, Hopkins J (1993) Evidence for the expression of two distinct MHC class II DRb like molecules in the sheep. *Animal Genetics*. in press.

Dutia BM, Hopkins J (1991) Analysis of the CD1 cluster in sheep. *Vet Immunol Immunopathol* **27**:189-194

Eastgate JA, Symons JA, Wood NC, Grinlinton FM, di Giovine FS, Duff GW (1988) Correlation of plasma interleukin-1 levels with disease activity in rheumatoid arthritis. *Lancet* **2**:706-709

Eastgate JA, Symons JA, Wood NC, Capper SJ, Duff GW (1991) Plasma levels of interleukin-1-alpha in rheumatoid arthritis. *Br J Rheumatol* **30**:295-297

Egner W, Prickett TCR, Hart DNJ (1992) Dendritic cells, monocytes and macrophages: A comparison of adhesion molecule repertoire. *2nd Int Symposium on Dendritic cells in fundamental and clinical immunology,Amsterdam, The Netherlands. Abstracts,* p15.

Eisenberg SP, Brewer MY, Verderber E, Heimdal P, Brandhuber BJ, Thompson RC (1991) Interleukin 1 receptor antagonist is a member of the interleukin 1 gene family: Evolution of a cytokine control mechanism. *Proc Natl Acad Sci USA* **88**:5232-5236

Eisenberg SP, Evans RJ, Arend WP, Verderber E, Brewer MT, Hannum CH, Thompson RC (1990) Primary structure and functional expression from complementary DNA of a human interleukin-1 receptor antagonist. *Nature* **343**:341-346

Elias JA, Gustilo K, Beader W, Freundlich B (1987) Synergistic stimulation of fibroblast prostaglandin production by recombinant interleukin 1 and tumor necrosis factor. *J Immunol* **138**:3812-3816

Endres S, Cannon JG, Ghorbani R, Dempsey RA, Sisson SD, Lonnemann G, Van der Meer JW, Wolff SM, Dinarello CA (1989). In vitro production of IL-1 beta, IL-1 alpha, TNF and IL-2 in healthy subjects: distribution, effect of cyclooxygenase inhibition and evidence of independent gene regulation. *Eur J Immunol* **19**:2327-2333

Endres S, Fulle J-H, Sinha B, Stoll D, Dinarello CA, Gerzer R, Weber PC (1991) Cyclic nucleotides differentially regulate the synthesis of tumour necrosis factor-α and interleukin-1β by human mononuclear cells. *Immunology* **72**:56-60

Enk AH, Katz SI (1992) Identification and induction of keratinocyte-derived interleukin-10. *J Immunol* **149**:92-95

Enk AH, Angeloni VL, Udey MC, Katz SI (1993) An essential role for Langerhans cell-derived IL-1 beta in the initiation of primary immune responses in skin. *J Immunol* **150**:3698-3704

Eriksson AE, Cousens LS, Weaver LH, Matthews BW (1991) Three-dimensional structure of human basic fibroblast factor. *Proc Natl Acad Sci USA* **88**:3441-3445

Espel E, Fromental C, Reichenbach P (1990) Activity and interleukin-1 responsiveness of SV40 enhancer motifs in a rodent immature T cell line. *EMBO J* **9**:929-937

Esser R, von Briesen H, Brugger M, Ceska M, Glienke W, Muller S, Rehm A, Rubsamen-Waigmann H, Andreesen R (1991) Secretory repertoire of HIV infected human monocytes/ macrophages. *Pathobiology* **59**:219-222

Eugui E, Almquist S (1990) Antibodies against membrane interleukin-1α activate accessory cells to stimulate proliferation of T-lymphocytes. *Proc Natl Acad Sci USA* **87**:1305-1309

Everson MP, Koopman WJ, Beagley KW (1992) Divergent T-cell cytokine profiles induced by dendritic cells from different tissues. *2nd Int symposium on dendritic cells in fundamental and clinical immunology, Amsterdam, The Netherlands. Abstracts*, p11

Fagarasan MO, Bishop JF, Rinaudo MS, Axelrod J (1990) Interleukin-1 induces early protein phosphorylation and requires only a short exposure for late-induced secretion of b-endorphin in a mouse pituitary cell line. *Proc Natl Acad Sci USA* **87**:2555-2559

Faherty DA, Claudy V, Plocinski JM, Kaffka K, Kilian P, Thompson RC, Benjamin WR (1992) Failure of IL-1 receptor antagonist and monoclonal antibody to inhibit antigen-specific immune responses *in vivo*. *J Immunol* **148**:766-771

Falk W, Van Hogen I, Krammer PH (1989) Activation of T cells by interleukin 1 involves internalisation of interleukin 1. *Lymphokine Res* **8**:263-268

Farrar WL, Kilian PL, Ruff MR, Hill JM, Pert CB (1988) Characterisation of interleukin 1 receptors in brain. *Adv Biochem Psychopharmacol* **44**:35-44

Farrar WL, Garcia Garcia G, Evans G, Michiel D, Linnekin D (1990) Cytokine regulation of protein phosphorylation. *Cytokine* **2:77-91**

Fasano MB, Cousart S, Neal S, McCall CE (1991) Increased expression of the interleukin 1 receptor on blood neutrophils of humans with the sepsis syndrome. *J Clin Invest* **88**:1452-1459

Feinberg AP, Vogelstein B (1984) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**:6-13

Ferreira SH, Lorenzetti BB, Bristow AF, Poole S (1988) Interleukin-1β as a potent hyperalgesic agent antagonised by a tripeptide analogue. *Nature*(London) **334**:698-700

Fiskerstrand C, Sargan D (1990) Nucleotide sequence of ovine interleukin-1 beta. *Nucl Acids Res* **18**:1765

Fiskerstrand CE, Roy DJ, Green I, Sargan DR (1992) Cloning, expression and characterisation of ovine interleukins-1α and β. *Cytokine* **4**:418-428

Folks TM, Jestement J, Kinter A, Dinarello CA, Fauci AS (1987) Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science* **238**:800-802

Fossum S (1989) Lymph-borne dendritic leukocytes do not recirculate, but enter the lymph node paracortex to become interdigitating cells. *Scand J Immunol* **27**:97-105

Frisch SM, Ruley HE (1987) Transcription form the stromelysin promoter is induced by interleukin-1 and repressed by dexamethasone. *J Biol Chem* **262**:16300-16304

Fuhlbrigge RC, Fine SM, Unanue ER, Chaplin (1988) Expression of membrane interleukin 1 by fibroblasts transfected with murine pro-interleukin-1 alpha cDNA. *Proc Natl Acad Sci USA* **85**:5649-5653

Fuhlbrigge RC, Hogquist KA, Unanue ER, Chaplin DD (1989) Molecular biology and genetics of IL-1. pp21-37, in*The year in Immunology 1988-1989,Basel, Switzerland.Publ Karger*

Furutani Y, Notake M, Yamaoshi M, Yamagishi J, Momura H, Ohue M, Furuta R, Fukui T, Yamada M, Nakamura S (1985) Cloning and characterisation of the cDNAs for human and rabbit interleukin-1 precursor (published erratum appears in Nucl Acids Res 1986 **14**:5142). *Nucleic Acids Res* **13**:5869-5882

Furutani Y, Notake M, Fukui T, Ohue M, Nomura H, Yamada M, Nakamura S (1986) Complete nucleotide sequence of the gene for human interleukin 1 alpha. *Nucleic Acids Res* **14**:3167-3179

Gaffney EV, Koch G, Tsai SC, Loucks T, Lingenfelter SE (1988) Correlation between human cell growth response to interleukin 1 and receptor binding. *Cancer Res* **48**:5455-5459

Gajewski TF, Pinnas M, Wong T, Fitch FW (1991) Murine Th1 and Th2 clones proliferate optimally in response to distinct antigen-presenting cell populations. *J Immunol* **146**:1750-1758

Gallay P, Mach JP, Carrel S (1991) Characterisation and detection of naturally occurring antibodies against IL-1 alpha and IL-1 beta in normal plasma. *Eur Cytokine Netw* **2**:329-338

Gallis B, Prickett SK, Jackson J, Slack J, Schooley K, Sims JE, Dower SK (1989) IL-1 induces rapid phosphorylation of the IL-1 receptor. *J Immunol* **143**:3235-3240

Gehrke L, Jobling SA, Paik LS, McDonald B, Rosenwasser LJ, Auron PE (1990) A point mutation uncouples human interkeukin-1 beta biological activity and receptor binding. *J Biol Chem* **265**:5922-5925

Geiger T, Rordorf C, Galakatos N, Seliogmann B, Henn R, Lazdins J, Vosbeck K (1992) Recombinant human C5a induces transcription but not translation of interleukin-1β mRNA in human monocytes. *Res Immunol* **143**:117-123

Gery I, Gershon RK, Waksman BH (1971) Potential of cultured mouse thymocyte responses by factors released by peripheral leucocytes. *J Immunol* **107**:1778-1780

Gery I, Gershon RK, Waksman BH (1972) Potentiation of the T-lymphocyte response to mitogens. I. The responding cell. *J Exp Med* **136**:128-142

Gery I, Waksman BH (1972) Potentiation of the T-lymphocte response to mitogens. II. The cellular source of potentiating mediator(s). *J Exp Med* **136**:143-155

Ghiara P, Scapigliati G, Tagliabue A, Boraschi D (1989) Characterisation of receptors for IL-1α and IL-1β on lymphoid and non-lymphoid cells. *J Immunol Res* **1**:13

Ghosh S, Baltimore D (1990) Activation in vitro of NF-kB by phosphorylation of its inhibitor IkB. *Nature* **344**:678-682

Gieseler RKH, Xu H, Peters JH (1992) Serum-free differentiation of rat and human dendritic cells, accompanied by acquisition of the nuclear laminins A/C as differentiation markers. *2nd Int Symposium on Dendritic cells in fundamental and clinical immunology, Amsterdam, The Netherland. Abstracts*, p61

Gilmour JEM, Senior JM, Burns NR, Esnouf MP, Gull K, Kingsman SM, Kingsman AJ, Adams SE (1989) A novel method for the purification of HIV-1 p24 protein from hybrid Ty virus-like particles (Ty-VLPs). *AIDS* **3**:717-723

Giri J, Lomedico PT, Mizel S (1985) Studies on the synthesis and secretion of interleukin 1. I. A 33,000 molecular weight precursor for interleukin 1. *J Immunol* **134**:343-349

Gonzales C (1991) MPhil Thesis, University of Edinburgh.

Goodman MG (1989) Induction of interleukin 1 activity from macrophages by direct interaction with C8-substituted guanine ribonucleosides. *Int J Pharmacol* **10**:579-586

Gowen M, Mundy GR (1986) Actions of recombinant interleukin 1, interleukin 2 and interferon-γ on bone resorption in vitro. *J Immunol* **136**:2478-2482

Gowen M, Chapman K, Littlewood A, Hughes D, Evans D, Russell G (1990) Production of tumour necrosis factor by human osteoblasts is modulated by other cytokines but not by osteotropic hormones. *Endocrinol* **126**:1250-1255

Granowitz EV, Clark BD, Mancilla J, Dinarello CA (1991) Interleukin-1 receptor antagonist competitively inhibits the binding of interleukin-1 to the type II interleukin-1 receptor. *J Biol Chem* **266**:14147-14150

Granowitz EV, Porat R, Mier JW, Pribble JP, Stiles DM, Bloedow DC, Catalane MA, Wolff SM, Dinarello CA (1992a) Pharmacokinetics, safety and immunomodulatory effects of human recombinant interleukin-1 receptor antagonist in healthy humans. *Cytokine* **4**:353-360

Granowitz EV, Vannier E, Poutsakia DD, Dinarello CA (1992b) Effect of interleukin-1 (IL-1) blockade on cytokine synthesis: II IL-1 receptor antagonist inhibits lipopolysaccharide-induced cytokine synthesis by human monocytes. *Blood* **79**:2364-2369

Graves BJ, Hatada MH, Hendrickson WA, Miller JK, Madison VS, Satow Y (1990) Structure of interleukin 1α at a 2.7A resolution. *Biochem* **29**:2679-2684

Gray PW, Glaister D, Chen E, Goeddel DV, Pennica D (1986) Two interleukin 1 genes in the mouse:cloning and expression of the c-DNA for murine interleukin 1β. *J Immunol* **137**:3644-3648

Green I, Sargan D (1991) Sequence of the cDNA encoding ovine tumor necrosis factor-α: problems with cloning by inverse PCR. *Gene* **109**:203-210

Green IR, Fiskerstrand C, Bertoni G, Roy DJ, Peterhans E, Sargan D (1993) Expression and characterisation of bioactive recombinant ovine TNFα: Some species specificity in cytotoxic responses to TNF. *Cytokine* **5**:in press

Grenfell S, Smithers N, Miller K, Solari R (1988) Receptor-mediated endocytosis and nuclear transport of human interleukin 1α. *Biochem J* **264**:813-822

Grenfell S, Smithers N, Miller K, Solari R (1989) Receptor-mediated endocytosis and nuclear transport of human interleukin 1 alpha. *Biochem J* **264**:813-822

Grenfell S, Smithers N, Witham S, Shaw A, Graber P, Solari R (1991) Analysis of mutations in the putative nuclear localisation sequence of interleukin-1 beta. *Biochem J* **280**:111-116

Gronenborn AM, Wingfield PT, McDonald HR, Schmeissner U, Clore GM (1988) Site directed mutants of human interleukin-1 alpha: a 1H-NMR and receptor binding study. *FEBS Lett* **231**:135-138

Grossman RM, Kruger J, Yourish D, Granelli-Piperno A, Murphy DP, May LT, Kupper TS, Sehal PB, Gottlieb A (1989) Interleukin-6 is expressed in high levels in psotiatic skin and stimulates proliferation of cultured keratinocytes. *Proc Natl Acad Sci USA* **86**:6367-6371

Groves RW, Ross E, Barker JN, Ross JS, Camp RD, MacDonald DM (1992) Effect of in vivo interleukin-1 on adhesion molecule expression in normal human skin. *J Invest Dermatol* **98**:384-387

Gruaz-Chatellard D, Baumberger C, Saurat JH, Dayer JM (1991) Interleukin-1 receptor antagonist in human epidermis and cultured keratinocytes. *FEBS Lett* **294**:137-140

Gubler U, Hoffman BJ (1983) A simple and very efficient method for generating cDNA libraries. *Gene* **25**:263-269

Gubler U, Chua AO, Stern AS, Hellmann CP, Vitek MP, DeChiara TM, Benjamin WR, Collier KJ, Dukovich M, Familietti PC et al. (1986) Recombinant human interleukin 1α: Purification and biological characterisation. *J Immunol* **136**:2492-2497

Gurney AL, Park EA, Giralt M, Liu JS, Hanson RW (1992) Opposing actions of Fos and Jun on transcription of the phoshpoenol pyruvate carboxykinase (GTP) gene - Dominant negative regulation by Fos. *J Biol Chem* **267**:18133-18139

Guy GR, Chua SP, Wong NS, Ng SB, Tan YH (1991) Interleukin 1 and tumour necrosis factor activate common multiple protein kinases in human fibroblasts. *J Biol Chem* **266**:14343-14352

Habazetti J, Gondol D, Wiltscheck R, Otlewski J,Schleicher M, Holak TA (1992) Structure of hisactophilin is similar to interleukin-1 beta and fibroblast growth factor. *Nature* **359**:855-858

Hall JG (1987) Studies of the cells in the afferent and efferent lymph of lymph nodes draining a site of skin homografts. *J Exp Med* **125**:737-754

Hammerberg C, Fisher G, Voorhees JJ, Cooper KD (1990) Human epidermis processes both IL-1 alpha and IL-1beta into novel molecular isoforms. *J Invest Dermatol* **94**:532A

Hammerberg C, Arend WP, Fisher GJ, Chan LS, Berger AE, Haskill JS (1992) Interleukin-1 receptor antagonist in normal and psoriatic epidermis. *J Clin Invest* **90**:571-583

Hammonds P, Begg M, Beresford G, Espinal J, Clarke J, Mertz RJ (1990) Insulin-secreting beta-cells possess specific receptors for interleukin-1 beta. *FEBS Lett* **261**:97-100

Hannum CH, Wilcox CJ,Arend WP,Joslin FG, Dripps DJ, Heimdal PL, Armes LG, Sommer A, Eisenberg SP, Thompson RC (1990) Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. *Nature* **343**:336-340

Harkiss GD, Price K, Veitch D (1989) Partial purification and characterisation of a factor produced by mitogen-stimulated sheep mononuclear cells with thymocyte-activating and cartilage-degrading properties. *J Immunol Methods* **117**:321-241

Harkiss GD, Hopkins J, McConnell I (1990) Uptake of antigen by afferent lymph dendritic cells mediated by antibody. *Eur J Immunol* **20**:2367-2373

Hart PH, Cooper RL, Finlay-Lones (1991) IL-4 suppresses IL-1β, TNFα and PGE_2 production by human peritoneal macrophages. *Immunology* **72**:344-349

Hart PH, Jones CA, Jones KL, Finlay-Jones JJ (1993) Reduced secretion of IL-1β by peritoneal cells from patients on continuous ambulatory peritoneal dialysis. *Immunol Cell Biol* **71**:99-107

Haskill S, Martin G, Van Le L, Morris J, Peace A, Bigler CF, Jaffe GJ, Hammerberg C, Sporn SA, Fong S et al (1991) cDNA cloning of an intracellular form of the human interleukin-1 receptor antagonist associated with epithelium. *Proc Natl Acad Sci* **88**:3681-3685

Hawrylowicz CM, Santoro SA, Platt FM, Unanue ER (1989a) Activated platelets express IL-1 activity. *J Immunol* **143**:4015-4018

Hawrylowicz CM, Duncan LM, Fuhlbrigge RC, Unanue ER (1989b) Regulation of antigen presentation. II Anti-Ig and IL-2 induce IL-1 production by murine splenic B cells. *J Immunol* **142**:3361-3368

Hawrylowicz CM, Howells GL, Feldman M (1991) Platelet-derived interleukin 1 induces human endothelial adhesion molecule expression and cytokine production. *J Exp Med* **174**:785-790

Hazuda DJ, Lee JC, Young PR (1988) The kinetics of Interleukin 1 from activated monocytes. Differences between interleukin 1α and interleukin 1β. *J Biol Chem* **263**:8473-8479

Hazuda DJ, Strickler J, Kueppers F, Simon PL, Young PR (1990) Processing of precursor interleukin-1 beta and inflammatory disease. *J Biol Chem* **265**:6318-6322

Hazuda DJ, Strickler J, Simon P, Young PR (1991) Structure-function mapping of interleukin 1 precursors. Cleavage leads to a conformational change in the mature protein. *J Biol Chem* **266**:7081-7086

Heckmann M, Adelmann-Grill BC, Hein R, Kreig T (1993) Biphasic effects of interleukin-1α on dermal fibroblasts: Enhancement of chemotactic responsiveness at low concentrations and of mRNA expression for collagenase at high concentrations. *J Invest Dermatol* **100**:780-784

Heguy A, Baldar C, Bush K, Nagele R, Newton RC, Robb RJ, Horuk R, Telford AF, Melli M (1991) Internalisation and nuclear localisation of interleukin 1 are not sufficient for function. *Cell Growth Different* **2**:311-315

Heguy A, Baldari CT, Macchia G, Telford JL, Melli M (1992) Amino acids conserved in interleukin-1 receptors (IL-1Rs) and the Drosophila toll protein are essential for IL-1R signal transduction. *J Biol Chem* **267**:2605-2609

Hein WR, Dudler L, Marston WL, Hopkins J, Dutia BM, Keech K, Brandon MR, Mackay CR (1991) Summary of workshop findings for leukocyte antigens of sheep. *Vet Immunol Immunopathol* **27**:28-30

Henderson B, Thompson RC, Hardingham T, Lewthwaite J (1991) Inhibition of interleukin-1 induced synovitis and articular cartilage proteoglycan loss in the rabbit knee by recombinant human interleukin-1 receptor antagonist. *Cytokine* **3**:246-249

Herman J, Dinarello CA, Kew MC, Rabson AR (1985) The role of interleukin 1 (IL-1) in tumor-NK cell interactions: correction of defective NK activity in cancer patients by treating target cells with IL-1. *J Immunol* **135**:2882-2886

Herzberg H, Blum B, Ronspeck W, Frenzel B, Brandt E, Ulmer AJ, Flad HD (1989) Functional and molecular characterisation of a monoclonal antibody against the 165-186 peptide of human IL-1 beta. *Scand J Immunol* **30**:549-562

Hession C, Decker JM, Sherblom AP, Kumar S, Yue CC, Mattaliano RJ, Tizard R, Kawashima E, Schmeissner U, Heletky S, et al (1987) Uromodulin (Tamm-Horsfall glycoprotein): a renal ligand for lymphokines. *Science* **237**:1479-1484

Heufler C, Koch F, Schuler G (1988) Granulocyte/macrophage colony-stimulating factor and interleukin 1 mediate the maturation of murine epidermal cells into potent immunostimulatory dendritic cells. *J Exp Med* **167**:700-705

Heufler C, Topar G, Koch F, Trochenbacher B, Kampgen E, Romani N, Schuler G (1992) Cytokine gene expression in murine epidermal cell suspensions: Interleukin 1β and macrophage inflammatory protein 1α are selectively expressed in Langerhans cells but are differentially regulated in culture. *J Exp Med* **176**:1221-1226

Higuchi CM, Thompson JA, Lindgren CG, Gillis S, Widmer MB, Kern DE, Fefer A (1989) Induction of lymphokine-activated killer activity by IL-4 in human lymphocytes preactivated by interleukin-2 in vivo or in vitro. *Cancer Res* **49**:6487-6492

Hill S, Griffiths S, Kimber I, Knight SC (1992) Migration of dendritic cells during contact sensitisation. *2nd Int Symposium on Dendritic cells in fundamental and clinical immunology,Amsterdam, The Netherlands. Abstracts*, p74

Hinnen A, Hicks JB, Fink GR (1978) Transformation of yeast. *Proc Natl Acad Sci USA* **75**:1929-1933

Hogquist KA, Unanue ER, Chaplin DD (1991a) Release of IL-1 from mononuclear phagocytes. *J Immunol* **147**:2181-2186

Hogquist KA, Nett MA, Unanue ER, Chaplin DD (1991b) Interleukin-1 is processed and released during apoptosis. *Proc Natl Acad Sci USA* **88**:8485-8489

Hollis DE, Lyne AG (1972) Acetylcholinesterase-positive Langerhans cells in the epidermis and wool follicles of the sheep. *J Invest Dermatol* **58**:211-217

Hopkins J, Dutia BM, Bujdoso R, McConnell (1989) In vivo modulation of CD1 and MHC class II expression by sheep afferent lymph dendritic cells. *J Exp Med* **170**:1303-1318

Hopkins SJ, Humphreys M, Kinnaird A, Jones DA, Kimber I (1990) Production of interleukin-1 by draining lymph node cells during the induction phase of contact sensitisation in mice. *Immunology* **71**:493-496

Horuk R, Huang JJ, Covington M, Newton RC (1987) A biochemical and kinetic analysis of the interleukin-1 receptor. Evidence for differences in molecular properties of IL-1 receptors. *J Biol Chem* **262**:16275-16278

Horuk R, McCubrey J (1989) The interleukin-1 receptor in Raji human B-lymphoma cells. Molecular characterisation and evidence for receptor-mediated activation of gene expression. *Biochem J* **260**:657-663

Horuk R (1991) Differences in internalisation and intracellular processing of interleukin-1 associated with the two forms of the interleukin-1 receptor found in B-cells and T-cells. *Biochem J* **273**:79-83

Hosoi J, Murphy GF, Egan CL, Lerner EA, Grabbe S, Asahina A, Granstein RD (1993) Regulation of Langerhans cell function by nerves containing calaitonin gene-related peptide. *Nature* **363**:159-163

Howard AD, Kostura MJ, Thornberry N, Ding GJF, Limjuco G, Weidner JPS, Hogquist KA, Chaplin DD, Mumford RA, Schmidt JA, Tocci MJ (1991) IL-1 converting enzyme requires aspartic acid residues for processing of IL-1β precursor at two distinct sites and does not cleave the 31-kDa IL-1α. *J Immunol* **147**:2964-2969

Hoyne GF, Callow MG, Kuo M-C, Thomas WR (1993) Comparison of antigen presentation by lymph node cells from protein and peptide-primed mice. *Immunol* **78**:58-64

Huang JJ, Newton RC, Rutledge SJ, Horuk R, Matthew JB, Covington M, Lin Y (1988) Characterisation of murine IL-1 beta. Isolation, expression and purification. *J Immunol* **140**:3838-3843

Hudson L, Hay FC (1989) Non-specific esterase stain for macrophages. *Practical Immunology 3rd edition,* Blackwell Scientific Publications. p28

Hunninghake GW, Monks BG, Geist LJ, Monick MM, Monroy MA, Stinski MF, Webb AC, Dayer JM, Auron PE, Fenton MJ (1992) The functional importance of a cap site-proximal region of the human prointerleukin 1β gene is defined by viral protein *trans*-activation. *Mol Cell Biol* **12**:3439-3448

Hunninghake GW (1984) Release of interleukin-1 by alveolar macrophages of patients with active pulmonary sarcoidosis. *Ann Rev Resp Dis* **129**:569-572

Huntley JF, Newlands GF, Jackson F, Miller HR (1992) The influence if challenge dose, duration of immunity, or steroid treatment on mucosal mast cells and on the distribution of sheep mast cell proteinase in Haemonchus-infected sheep. *Parasite Immunol* **14**:429-440

Hurme M, Serkkola E (1991) Different signals are required for the expression of interleukin-1 alpha and beta genes in human monocytes. *Scand J Immunol* **33**:713-718

Hurme M (1987) Membrane-associated interleukin 1 is required for the activation of T cells in the anti-CD3 antibody-induced T cell response. *J Immunol* **139**:1168-1172

Ikejima T, Clark BD, Mancilla J, Sirko S, Dinarello CA (1990) Affinity purified antibodies against a 17 amino acid portion of murine IL-1R recognises 80kDa (IL-1RtI) peptide and blocks differentially IL-1α and IL-1β binding on D10S cells. *Lymph Res* **9**:583-

Inaba K, Steinman RM (1984) Resting and sensitised T lymphocytes exhibit distinct stimulatory (and antigen presenting cell) requirements for growth and lymphokine release. *J Exp Med* **160**:1717-1735

Inaba K, Witmer-Pack MD, Inaba M, Muramatsu S, Steinman R (1988) The function of Ia^+ dendritic cells and Ia^- dendritic cell precursors in thymocyte mitogenesis to lectin and lectin plus interleukin 1. *J Exp Med* **167**:149-162

Inaba K, Steinman RM, Witmer-Pack M, Aya H, Inaba M, Sudo T, Wolpe S, Schuler G (1992) Identification of proliferating dendritic cell precursors in mouse blood. *J Exp Med* **175**:1157-1167

Innis MA, Myambo KB, Gelfand DH, Brown MA (1988) DNA sequencing with Thermus aquaticus DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proc Natl Acad Sci USA* **85**:9436-9440

Isono N, Kumagai K (1989) Production of interleukin-1 inhibitors by the murine macrophage cell line P388D which produces interleukin-1. *Microbiol Immunol* **33**:43-57

Iwamoto GK, Monick MM, Clark SD, Auron PE, Stinski MF, Hunninghake GW (1990) Modulation of interleukin 1 beta gene expression by the immediate early genes of cytomegalovirus. *J Clin Invest* **85**:1853-1857

Janson RW, Hance KR, Arend WP (1991) Production of IL-1 receptor antagonist by human in vitro-derived macrophages. Effects of lipopolysaccharide and granulocyte-macrophage-colony-stimulating factor. *J Immunol* **147**:4218-4223

Jensen J, Schultz RD (1991) Effect of infection by bovine viral diarrhoea virus (BVDV) in vitro on interleukin-1 activity of bovine monocytes. *Vet Immunol Immunopathol* **29**:251-265

Jobling SA, Auron PE, Gurka G, Webb AC, McDonald B, Rosenwasser LJ, Gehrke L (1988) Biological activity and receptor binding of human prointerleukin-1 beta and subpeptides. *J Biol Chem* **263**:16372-16378

Jones A, Geczy CL (1990) Thrombin and Factor Xa enhance the production of interleukin-1. *Immunol* **71**:236-241

Ju G, Labriola-Tompkins E, Campen CA, Benjamin WR, Karas J, Plocinski J, Biondi D, Kaffka KL, Kilian PL, Eisenberg SP, Evans RJ (1991) Conversion of the interleukin 1 receptor antagonist into an agonist by site-specific mutagenesis. *Proc Natl Acad Sci USA* **88**:2658-2662

Juhlin L, Shelley WB (1977) New staining techniques for the Langerhans cell. *Acta Dermato-venereol* **57**:289-296

Kahn AJ, Partridge NC (1987) New concepts in bone remodelling: an expanding role for the osteoblast. *Am J Otolaryngol* **8**:258-264

Kampgen E, Koch N, Koch F, Stoger P, Heufler C, Schuler G, Romani N (1991) Class II MHC complex molecules of murine dendritic cells: synthesis of invariant chain and antigen processing capacity are down regulated upon culture. *Proc Natl Acad Sci USA* **88**:3014-3018

Kampgen E, Lenz A, Koch F, Mikolajewski C, Hartmann AA, Romani N, Schuler G (1992) Interleukin-1 receptors on epidermal Langerhans' cells and lymphoid dendritic cells. *2nd Int symposium on dendritic cells in fundamental and clinical immunology, Amsterdam, The Netherlands. Abstracts*, p44

Kampschmidt RF (1981) Leukocyte endogenous mediator/endogenous pyrogen. In: Powande MC, Canonico PG, eds. The physiologic and metabolic responses of the host. Amsterdam: Elsevier/North Holland. p55-74

Kaplan G, Nusrat A, Witmer MD, Nath I, Cohn ZA (1987) Distribution and turnover of Langerhans cells during delayed immune responses in human skin. *J Exp Med* **165**:763-776

Kaplan J, Nielsen ML (1979) Analysis of macrophage surface receptors. I. Binding of α-macroglobulin-protease complexes to rabbit alveolar macrophages. *J Biol Chem* **254**:7323-7328

Kapsenberg ML, Stiekema F, Leene W (1985) Differential requirements for rabbit dendritic cells and macrophages in T lymphocyte proliferation induced by various mitogens. *Immunology* **55**:301-310

Kashiwado T, Oppenheimer-Marks N, Ziff M (1989) T cell inhibitor secreted by macrophages and endothelial cells. *Clin Immunol Immunopathol* **53**:137-150

Katsura G, Gottschall PE, Amimura A (1988) Identification of a high afinity receptor for interleukin-1 beta in rat brain. *Biochem Biophys Res Commun* **156**:61-67

Katsuura G, Gottschall PE, Arimura A (1988) Identification of a high-affinity receptor for inteleukin-1 beta in the rat brain. *Biochem Biophys Res Commun* **156**:61-67

Katz SI, Tamaki K, Sachs DH (1979) Epidermal Langerhans cells are derived from cells oroginating in bone marrow. *Nature* **282**:324-326

Kaur P, Saklatvala J (1988) Interleukin 1 and tumor necrosis factor increase phosphorylation of fibroblast proteins. *FEBS Lett* **241**:6-10

Kaur P, Welch WJ, Saklatvala J (1989) Interleukin 1 and tumor necrosis factor increase phosphorylation of the small heat shock protein hsp27. Effects in fibroblasts, Hep G2 and U937 cells. *FEBS Lett* **258**:269-273

Kawashima H, Yamagishi J, Yamayoshi M, Ohue M, Fukui T, Kotani H, Yamada M (1992) Structure-activity relationships in human interleukin-1α: identification of key residues for expression of biological activities. *Protein Eng* **5**:171-176

Kaye J, Procelli S, Tite J, Jones B, Janeway CA (1983) Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen-presenting cells in the activation of T cells. *J Exp Med* **158**:836-856

Kelly RH, Balfour BM, Armstrong JA, Griffiths S (1978) Functional anatomy of lymph nodes. II. Peripheral lymph-borne mononuclear cells. *Anat Rec* **190**:5-22

Kelly RH (1970) Localisation of afferent lymph cells within the draining node during a primary immune response. *Nature* **227**:510-513

Kessler DJ, Duyao MP, Spicer DB, Sonenshein GE (1992) NF-kB-like factors mediate interleukin 1 induction of c-myc gene transcription in fibroblasts. *J Exp Med* **176**:787-792

Killar LM, Hatfield CA, Carding SR, Pan M, Winterrowd GE, Bottomly K (1989) In vivo administration of interleukin 1 elicits increased Ia antigen expression on B cells through the induction of interleukin 4. *Eur J Immunol* **19**:2205-2210

Kingsman AJ, Kingsman SM (1988) Ty: A retroelement moving forward. *Cell* **53**:333-335

Kirkham B (1991) Interleukin-1, immune activation pathways and different mechanisms in osteoarthritis and rheumatoid arthritis. *Ann Rheum Dis* **50**:395-400

Klarnet JP, Kern DE, Dower SK, Matis LA, Cheever MA, Greenberg PD (1989) Helper-independent $CD8^+$ cytotoxic T lymphocytes express IL-1 receptors and require IL-1 for secretion. *J Imunol* **142**:2187-2191

Klinkert WEF, Steffen P (1988) In LB Schook and JG Tew (eds): *Antigen presenting cells: Diversity, differentiation and regulation.* New York: Alan Liss: pp251-257

Knoblock KF, Canning PC (1992) Modulation of in vitro porcine natural killer cell activity by recombinant interleukin-1α, interleukin-2 and interleukin-4. *Immunol* **76**:299-304

Knowles DM, Hoffman T, Ferrarini M, Kunkel HG (1978) The demonstration of acid-naphthyl acetate esterase activity in human lymphocytes: usefulness as a T cell marker. *Cell Immunol* **35**:112-123

Knudsen , Dinarello CA, Strom TB (1986) Prostaglandins posttranscriptionally inhibit monocyte expression of interleukin 1 activity by increasing intracellular cyclic adenosine monophosphate. *J Immunol* **137**:3189-3194

Knudsen PJ, Dinarello CA, Strom TB (1987) Glucocorticoids inhibit trasnscriptional and post-transcriptional expression of interleukin 1 in U937 cells. *J Immunol* **139**:4129-4134

Kobayashi M, Imamura M, Gotohda Y, Maeda S, Iwasaki H, Sakurada K, Kasai M, Hapel AJ, Miyazaki T (1991) Synergistic effects of interleukin-1β and interleukin-3 on the expansion of human hematopoetic progenitor cells in liquid cultures. *Blood* **78**:1947-1953

Kobayashi N, Hamamoto Y, Koyanagi Y, Chen IS, Yamamoto N (1989) Effect of interleukin-1 on the augmentation of human immunodeficiency virus gene expression. *Biochem Biophys Res Commun* **165**:715-721

Kobayashi Y, Yamamoto K, Saido T, Kawasaki H, Oppenheim JJ, Matsushima K (1990) Identification of a calcium-activated neutral protease as a processing enzyme for human interleukin-1 alpha. *Proc Natl Acad Sci USA* **87**:5548-5552

Koch KC, Ye K, Clark BD, Dinarello CA (1992) Interleukin 4 upregulates gene and surface IL-1 receptor type I in murine T helper type 2 cells. *Eur J Immunol* **22**:153-157

Kohase M, Zhang YH, Lin JX, Yamazaki S, Sehgal PB, Vilcek J (1988) Interleukin-1 can inhibit interferon-beta synthesis and its antiviral action: comparison with tumor necrosis factor. *J Interferon Res* **8**:559-570

Koide S, Steinman RM (1987b) Induction of murine interkeukin 1: stimuli and responsive primary cells. *Proc Natl Acad Sci* **84**:3802-3806

Koide S, Steinman RM (1988) Induction of interleukin 1α mRNA during the antigen-dependent interaction of sensitised T lymphoblasts with macrophages. *J Exp Med* **168**:409-416

Koide SL, Inaba K, Steinman RM (1987a) Interleukin-1 enhances T-dependent immune responses by amplifying the function of dendritic cells. *J Exp Med* **165**:515-530

Kornbluth RS, Oh PS, Munis JR, Cleveland PH, Richman DD (1989) Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by human immunodeficiency virus in vitro. *J Exp Med* **169**:1137-1151

Kraal G, Breel M, Janse M, Bruin G (1986) Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognised by a monoclonal antibody. *J Exp Med* **163**:981-997

Krane SM, Dayer JM, Simon LS, Byrne S (1985) Mononuclear cell-conditioned medium containing mononuclear cell factor (MCF), homologous with interleukin 1, stimulates collagen and fibronectin synthesis by adherent rheumatoid synovial cells: Effects of prostaglandin E_2 and indomethacin. *Collagen Relat Res* **5**:99-117

Kreeger JM, Snider TG, Olcott BM (1991) Spontaneous murine thymocyte comitogenic activity consistent with interleukin-1 in cattle naturally infected with *Mycobacterium paratuberculosis*. *Vet Immunol Immunopathol* **28**:317-326

Krieg PA, Melton DA (1984) Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucl Acid Res* **12**:7057-7070

Krieger JI, Chesnut RW, Grey HM (1986) Capacity of B cells to function as stimulators of a primary mixed leukocyte reaction. *J Immunol* **137**:3117-3123

Kroggel R, Martin M, Pingoud V, Dayer JM, Resch K (1988) Two-chain structure of the interleukin 1 receptor. *FEBS Lett [EUH]* **229**:59-62

Krogh Rasmussen A, Kayser L, Bech K, Feldt-Rasmussen U, Perrild H, Bendtzen K (1991) Influence of interleukin 6 on the function of secondary cultures of human thyrocytes. *Acta Endocrinol (Copenh)* **124**:577-582

Kronheim SR, Cantrell MA, Deeley MC, March C, Glakin P, Anderson T, Hemenway T, Merriam J, Cosmam D, Hopp T (1986) Purification to homogeneity of IL-1 beta protein. *Biotechnology* **4**:1078-1082

Kruys V, Marinx O, Shaw G, Deschamps J, Huez G (1989) Translational blockade imposed by cytokine-derived UA-rich sequences. *Science* **245**:852-855

Kuhweide R, Van Damme J, Lorre K, Baroja ML, Tsudo M, Ceuppens JL (1990) Accessory cell-derived helper signals in human T-cell activation with phythemagglutinin: induction of interleukin2, responsiveness of interleukin-6 and production of interleukin 2 by interleukin-1. *Cytokine* **2**:45-54

Kunkel SL, Spengler M, May M, Spengler R, Larrick J, Remick D (1988) Prostaglandin E2 regulates macrophage-derived tumor necrosis factor gene expression. *J Biol Chem* **263**: 5380-5384

Kupper TS, McGuire J (1986) Hydrocortisone reduces both constitutive and UV-elicited release of epidermal thymocyte activating factor (ETAF) by cultured keratinocytes. *J Invest Dermatol* **87**:570-573

Kupper TS, Lee F, Birchall N, Clark S, Dower S (1988) Interleukin 1 binds to specific receptors on human keratinocytes and induces granulocyte macrophage-stimulating factor mRNA and protein. A potential autocrine role for interleukin 1 in epidermis. *J Clin Invest* **82**:1787-1792

Kurt-Jones EA, Virgin HW IV, Unanue ER (1986) In vivo and in vitro expression of macrophage membrane interleukin 1 in response to soluble and particulate stimuli. *J Immunol***137**:10-14

Kyshe-Andersen J (1984) Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods* **10**:203-209

Labadia M, Faanes RB, Rothlein R (1990) Role of adherence in the induction of membrane-associated interleukin-1 on mouse peritoneal macrophages. *J Leukoc Biol* **48**:420-425

Labriola-Tompkins E, Chandran C, Kaffka KL, Biondi D, Graves BJ, Hatada M, Madison VS, Karas J, Kilian PL, Ju G (1991) Identification of the discontinuous binding site in human interleukin 1β for the type I interleukin 1 receptor. *Proc Natl Acad Sci* **88**:11182-11186

Lacey DL, Erdmann JM (1990) IL-1 and IL-4 modulate IL-1 receptor expression in a murine T cell line. *J Immunol* **145**:4145-4153

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685

Lafage M, Maroc N, Dubreuil P, de Waal Melefijt R, Pebusque MJ, Carcasonne Y, Mannoni P (1989) The interleukin-1 alpha gene is located on the long arm of chromosome 2 at band q13. *Blood* **73**:104-107

Lake BD (1971) Histochemical detection of the enzyme deficiency in blood films in Wolman's disease. *J Clin Pathol* **24**:617-620

LaMarre J, Wollenberg GK, Gonias SL, Hayes MA (1991) Cytokine binding and clearance properties of proteinase-activated alpha 2-macroglobulins. *Lab Invest* **65**:3-14

Larsen CP, Steinman RM, Witmer-Pack M, Hankins DF, Morris PJ, Austyn JM (1990) Migration and maturation of Langerhans cells in the skin transplants and explants. *J Exp Med* **172**:1483-1493

Le May LG, Otterness IG, Vander AJ, Kluger MJ (1990) In vivo evidence that the rise in plasma IL 6 following injection of a fever-inducing dose of LPS is mediated by IL 1 beta. *Cytokine* **2**:199-204

Le Moal MA, Stoeck M, Cavaillon JM, MacDonald HO, Truffa-Bachi P (1988) A sensitive, IL-2 dependent, assay for IL-1. *J Immunol Methods* **107**:23-30

Le S, Springer T, Haynes B, Singer K (1987) Anti LFA-3 monoclonal antibody induces interleukin 1 (IL-1) released by thymic epithelial (TE) cells and monocytes. *Fdn Proc Abstr* **46**:447

Lederer J, Czuprynski C (1992) Characterisation and identification of interleukin-1 receptors on bovine neutrophils. *J Leuk Biol* **51**: 586-590

Lederer J, Czuprynski C (1993) Characterisation and identification of interleukin-1 receptors on bovine fibroblasts. *Mol Immunol* **30**:191-196

Lederer JA, Czuprynski CJ (1989a) Production and purification of bovine monocyte-derived interleukin 1. *Vet Immunol Immunopathol* **23**:201-211

Lederer JA, Czuprynski CJ (1989b) Preference of bovine thymocytes and fibroblasts for bovine interleukin 1. *Vet immunol Immunopathol* **23**:213-222

Lee SC, Liu W, Dickson DW, Brosnan CF (1993) Cytokine production by human fetal microglia and astrocytes. *J Immunol* **150**:2659-2667

Lee SW, Tsou AP, Chan H, Thomas J, Petrie K, Eugui EM, Allison AC (1988) Glucocorticoids selectively inhibit the transcription of the interleukin 1 beta gene and decrease the stability of the interleukin 1 beta mRNA. *Proc Natl Acad Sci* **85**:1204-1208

LeMay LG, Otterness IG, Vander AJ, Kluger MJ (1990) In vivo evidence that the rise in plasma IL-6 following injection of a fever-inducing dose of LPS is mediated by IL-1 beta. *Cytokine* **2**:199-204

Leong SR, Flaggs GM, Lawman M, Gray PW (1988a) The nucleotide sequence for the cDNA of bovine interleukin-1 alpha. *Nucl Acid Res***16**:9053

Leong SR, Flaggs GM, Lawman M, Gray PW (1988b) The nucloetide sequence for the cDNA of bovine interleukin-1 beta. *Nucl Acid Res***16**:9054

Lerner UH, Ljunggren O, Dewhurst FE, Boraschi D (1991) Comparison of human interleukin-1 beta and its 163-171 peptide in bone resorption and the immune response. *Cytokine* **3**:141-148

Levine TP, Chain BM (1992) Endocytosis by antigen presenting cells: dendritic cells are as endocytically active as other antigen presenting cells. *Proc Natl Acad Sci USA* **89**:8342-8346

Limb GA, Hamblin AS, Wolstencroft RA, Dumonde DC (1991) Selective up-regulation of human granulocyte integrins and complement receptor 1 by cytokines. *Immunol* **74**:696-702

Lomedico PT, Gubler U, Hellman CP, Dukovich M, Giri JG, Pan Y-C, Coller K, Seminow R, Chua AO, Mizel SB (1984) Cloning and expression of murine interleukin-1 cDNA in Escherischia coli. *Nature* **312**:458-462

Lomedico PT, Gubler U, Mizel SB (1987) Cloning and expression of murine, human and rabbit interleukin 1 genes. *Lymphokines* **13**:139-150

Lonnemann G, Endres S, Van der Meer JW, Cannon JG, Koch KM, Dinarello CA (1989) Differences in the synthesis and kinetics of release of interleukin 1 alpha, interleukin 1 beta and tumor necrosis factor from human mononuclear cells. *Eur J Immunol* **19**:1531-1536

Loppnow H, Libby P (1992) Functional significance of human vascular smooth muscle cell-derived interleukin 1 in paracrine and autocrine regulation pathways. *Exp Cell Res* **198**:183-190

Lord PC, Wilmoth LM, Mizel SB, McCall CE (1991) Expression of interleukin-1 alpha and beta genes by human blood polymorphonuclear leukocytes. *J Clin Invest* **87**:1312-1321

Lovett DH, Martin M, Burstein S, Szamel M, Gemsa D, Resch K (1988) Interleukin-1 and the glomerular mesangium III. IL-1-dependent stimulation of mesangial cell protein kinase activity. *Kidney Int* **34**:26-35

Lowenthal JW, MacDonald HR (1986) Binding and internalisation of interleukin 1 by T cells. Direct evidence for high and low affinity classes of interleukin 1 receptor. *J Exp Med* **164**:1060-1074

Lowenthal JW, MacDonald HR (1987) Expression of interleukin 1 receptors is restricted to the L3T4+ subset of mature T lymphocytes. *J Immunol* **138**:1-3

Luger TA, Stadler BM, Katz SI, Oppenhein JJ (1981) Epidermal cell (keratinocyte)-derived thymocyte activating factor (ETAF). *J Immunol* **127**:1493-1498

Lundqvist EN, Back O (1990) Interleukin-1 decreases the number of Ia^+ epidermal dendritic cells but increases their expression of Ia antigen. *Acta Derm Venereol (Stockh)* **70**:391-394

Luqman M, Greenbaum L, Bottomly K (1992) Differential effect of interleukin 1 on naive and memory $CD4^+$ T cells. *Eur J Immunol* **22**:95-100

Lynn WA, Golenbock DT (1992) Lipopolysaccharide antagonists. *Immunology Today* **13**:271-276

Macatonia SE, Knight SC, Edwards AJ, Griffiths S, Fryer P (1987) Localisation of antigen on lymph node dendritic cells after exposure to the contact sensitiser fluorescein isothiocyanate. *J Exp Med* **166**:1654-1667

MacHugh HD, Bansaid A, Davis WC, Howard CJ, Parsons KR, Jones B, Kaushal A (1988) Characterisation of a bovine thymic differentiation antigen analogous to CD1 in the human. *Scand J Immunol* **27**:541-547

Mackay CR, Marston WL, Dudler L (1990) Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J Exp Med* **171**:801-817

Magnusson S, Petersen TE, Sottrup-Jensen L, Cleays H (1975) in *Proteases and biological control* Reich E, Rifkin D, Shaw E (eds).Cold Spring Harbor Laboratory,New York,pp 123-149

Mahe Y, Wakasugi H, Scamps C, Chouaib S, Tursz T (1991) Role of calcium on interleukin-1 production by monocytes: its relevance during T cell proliferation. *Lymphokine Cytokine Res* **10**:165-172

Maier JA, Voulalas P, Roeder D, Maciag T (1990) Extension of the life-span of human endothelial cells by an interleukin-1 alpha antisense oligomer. *Science* **249**:1570-1574

Malisewski CR, Baker PE, Schoenborn MA, Davis BS, Cosman D, Gillis S, Cerretti DP (1988) Cloning, sequencing and expression of bovine interleukin 1α and interleukin 1β complementary cDNAs. *Mol Immunol* **25**:429-437

Malisewski CR, Renshaw BR, Schoenborn MA, Urban JF, Baker PE (1990) Porcine IL-1α cDNA nucleotide sequence. *Nucl Acids Res* **18**:4282

Mancilla J, Ikejima I, Clark BD, Orencole SF, Sirko S, Dinarello CA (1989) Lectin binding suggests different glycosylation patterns of IL-1 receptors on different cells.*Cytokine* **1**:95-103

Maniatis T, Fritsch EF, Sambrook J (1982) *Molecular cloning: A laboratory manual.* Cold Spring Harbor, New York.

Manson J, Symons JA, di Giovine FS, Poole S, Duff GW (1989) Autoregulation of interleukin 1 production. *Eur J Immunol* **19**:261-165

Mantovani A, Dejana E (1989) Cytokines as communication signals between leukocytes and endothelial cells. *Immunology Today* **10**:370-375

March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, Gillis S, Henney CS, Kronheim SR, Grabstein K, Conlon PJ, Hopp TP, Cosman D (1985) Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature* **315**:641-648

Markwell MAK (1982) A new solid-state reagent to iodinate proteins. I. Conditions for the efficient labelling of antiserum. *Anal Biochem***125**:427-432

Marnell LL, Summers DF (1984) Characterisation of the small enzyme subunit, NS, of the vesicular stomatitis virus RNA polymerase. *J Biol Chem* **259**:13518-13524

Marucha PT, Zeff RA, Kreutzer DL (1991) Cytokine-induced IL-1β gene expression in the human polymorphonuclear leucocyte: Transcriptional and post-transcriptional regulation by tumor necrosis factor and IL-1. *J Immunol* **147**:2603-2608

Marusic A, Djikic I, Marusic M (1990) Cellular and morphological changes in lymphoid organs after a single injection of interleukin 1 alpha in the mouse. *Agents Actions* **31**:280-284

Maruyama T, Hoefsmit ECM, Kraal G (1992) A monoclonal antibody (MIDC-8) recognises an epitope of a dendritic cell specific endosomal deperminant involved in presentation of protein antigens. *2nd Int symposium on dendritic cells in fundamental and clinical immunology, Amsterdam, The Netherlands. Abstracts,* p7

Mason MJ, Van Epps DE (1989) In vivo neutrophil emigration in response to interleukin-1 and tumor necrosis factor-alpha. *J Leukoc Biol* **45**:62-68

Mathias S, Younes A, Kan C-C, Orlow I, Joseph C, Kolesnick RN (1993) Activation of the sphingomyelin signalling pathway in intact EL4 cells and in a cell-free system by IL-1β. *Science* **259**:519-522

Matsushima H, Roussel MF, Matsushima K, Hishinuma A, Sherr CJ (1991) Cloning, expression of murine interleukin-1 receptor antagonist in macrophages stimulated by colony-stimulating factor 1. *Blood* **78**:616-623

Matsushima K, Procopio A, Abe H, Scala G, Ortaldo JR, Oppenheim JJ (1985) Production of interleukin 1 activity by normal human peripheral blood B lymphocytes. *J Immunol* **135**:1132-1136

Matsushima K, Akahoshi T, Yamada M, Furutani Y, Oppenheim JJ (1986a) Properties of a specific interleukin (IL-1) receptor on human Epstein BarrVirus-transformed B lymphocytes: Identity of the receptor for IL 1-α and IL 1-β. *J Immunol* **136**:4496-4502

Matsushima K, Yodoi J, Tagaya Y, Oppenheim JJ (1986b) Down-regulation of interleukin 1 (IL 1) receptor expression by IL 1 and fate of internalised ^{125}I-labelled IL 1β in a human large granular lymphocyte cell line. *J Immunol* **137**:3183-3188

Maxfield FR, Willingham MC, Haigler Dragsten P, Pastan IH (1981) Binding, surface mobility, internalisation and degradation of rhodamine-labelled α_2-macroglobulin. *Biochemistry* **20**:5353

May SA, Hooke RE, Lees P (1990) The characterisation of equine interleukin-1. *Vet Immunol Immunopathol* **24**:169-175

McCollum R, Martel-Pelletier J, DiBatista J, Pelletier JP (1991) Regulation of interleukin 1 receptors in human articular chondrocytes. *JRheumatol Suppl* **27**:85-88

McDowell TL, Symons JA, Duff GW (1992) Sequence comparison and regulation in the 5' region of the human interleukin-1 alpha gene. *British Soc. Immunology, Spring meeting, Sheffield University. Abstracts,* :40(3.2)

McInnes CJ, Haig DM (1991) Cloning and expression of a cDNA encoding ovine granulocyte-macrophage colony-stimulating factor. *Gene* **105**:275-279

McIntyre KW, Stepan GJ, Kolinsky KD, Benjamin WR, Plocinski JM, Kaffka KL, Campen, CA, Chizzonite RA, Kilian PL (1991) Inhibition of interleukin-1 (IL-1) binding and bioactivity in vitro and modulation of acute inflammation in vivo by IL-1 receptor antagonist and anti-IL-1 receptor monoclonal antibody. *J Exp Med* **173**:931-939

McKenzie JL, Prickett TC, Hart DN (1989) Human dendritic cells stimulate allogeneic T cells in the absence of IL-1. *Immunology* **67**:290-297

McMahan CJ, Slack JL, Mosley B, Cosman D, Lupton SD, Brunton LL, Grubin CE, Wignall JM, Jenkins NA, Brannan CI et al. (1991) A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. *EMBO J* **10**:2821-2832

Mead DA, (1986). *Protein Engineering* **1**:67

Mellor J, Fulton SM, Dobson MJ, Wilson W, Kingsman SM, Kingsman AJ (1985a) A retrovirus-like strategy for expression of a fusion protein encoded by yeast transposon Ty 1. *Nature* **313**:243-246

Mellor J, Malim MH, Gull K, Tuite MF, McCready SM, Dibbayawan T, Kingsman SM, Kingsman AJ (1985b) Reverse transcriptase activity and Ty RNA are associated with virus-like particles in yeast. *Nature* **318**:583-586

Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, Green MR (1984) Efficient in vitro synthesis of biologically active RNA and RNA hybridisation probes from plasmids containing a bacteriophage SP6 promoter. *Nucl Acid Res* **12**:7035-7056

Meltzer MS, Skillman DR, Hoover DL, Hanson BD, Turpin JA, Kalter C, Gendelman HE (1990) Macrophages and the human immunodeficiency virus. *Immunol Today* 11:217-223

Meltzer MS, Baca L, Turpin JA, Kalter CD, Dieffenbach C, Friedman RM, Gendelman HE (1991) Regulation of cytokine and viral gene expression in monocytes infected with the human immunodeficiency virus. *Pathobiology* **59**:209-213

Mengozzi M, Bertini R, Sironi M, Ghezzi P (1991) Inhibition by interleukin-1 receptor antagonist of in vivo activities of interleukin-1 in mice. *Lymphokine Cytokine Res* **10**:405-407

Messing J (1979) A multipurpose cloning system based on single-stranded DNA bacteriophage M13. *Recomb DNA Tech Bull* **2**:43

Messing J (1983) In *Methods in Enzymol* **101**:27-78

Metcalf D (1989) The molecular control of cell division, differentiation commitment and maturation in haemopoetic cells. *Nature* **339**:27-30

Migita K, Eguchi K, Otsubo T, Kawami A, Nakao H, Ueki Y, Shimomura C, Kurata A, Fukuda T, Matsunaga et al (1991) Cytokine regulation of HLA on thyroid epithelial cells. *Clin Exp Immunol* **82**:548-552

Mills GB, May C, Hill M, Gelfand EW (1989) Role of protein kinase C in interleukin-1, anti-T3 and mitogenic lectin-induced interleukin-2 secretion. *J Cell Physiol* **141**:310-317

Mizel SB, Farrar JF (1979) Revised nomenclature for antigen-nonspecific T-cell proliferation and helper factors. *Cell Immunol* **48**:433-436

Mizel SB, Kilian PL, Lewis JC, Paganelli KA, Chizzonite RA (1988) The interleukin 1 receptor. Dynamics of interleukin 1 binding and internalisation in T cells and fibroblasts. *J Immunol* **138**:2906-2912

Mizutani H, Black R, Kupper TS (1991a) Human keratinocytes produce but do not process pro-interleukin-1 (IL-1) beta. Different strategies of IL-1 production and processing in monocytes and keratinocytes. *J Clin Invest* **87**:1066-1071

Mizutani H, Schechter N, Lazarus G, Black RA, Kupper TS (1991b) Rapid and specific conversion of precursor interleukin 1β (IL-1β) to an active species by human mast cell chymase. *J Exp Med* **174**:821-825

Mochizuki DY, Eisenman JR, Conlon PJ, Larsen, Tushinsky RJ (1987) Interleukin-1 regulates hematopoetic activity, a role previously ascribed to hemopoetin 1. *Proc Natl Acad Sci USA* **84**:5267-5271

Molina JM, Scadden DT, Amirault C, Woon A, Vannier E, Dinarello CA, Groopman JE (1990) Human immunodeficiency virus does not induce interleukin-1, interleukin-6 or tumor necrosis factor in mononuclear cells. *J Virol* **64**:2901-2906

Mollison KW, Mandecki W, Zuiderweg ERP, Fayer L, Fey TA, Krause RA, Conway RG, Miller L, Edjalji RP, Shallcross MA et al. (1989) Identification of receptor binding residues in the inflammatory complement protein C5a by site-directed mutagenesis. *Proc Natl Acad Sci USA* **86**:292-296

Monaco JJ (1992) A molecular model of MHC class-I-restricted antigen processing. *Immunology Today* **13**:173-179

Montz H, Koch KC, Zierz R, Gotze O (1991) The role of C5a in interleukin-6 production induced by lipopolysaccharide or interleukin-1. *Immunology* **74**:373-379

Moore MA, Warren DJ (1987) Synergy of interleukin 1 and granulocyte colony-stimulating factor: In vivo stimulation of stem-cell recovery and hematopoetic regeneration following 5-fluorouracil treatment of mice. *Proc Natl Acad Sci USA* **84**:7134-7137

Mooy P, Hoek A, de Haan-Meulman M, Drexhage HA (1992) Thyroid hormones enhance the capability of monocytes to mature into dendritic cells. Blocking effects of α-GM-CSF. *2nd Int Symposium on Dendritic cells in fundamental and clinical immunology, Amsterdam, The Netherlands. Abstracts*, p139

Morhenn VB, Lee SW, Ilnicka M, Eugui EM (1992) Activated human Langerhans cells expressmRNA for IL-1α and IL-1β and produce these cytokines but do not secrete them. *Cytokine* **4**:500-505

Mori S, Goto S, Okhawara S, Maeda K, Shimada K, Yoshinaga M (1988) Cloning and sequence analysis of a cDNA for lymphocyte proliferation potentiating factor of rabbit polymorphonuclear leukocytes: identification as a rabbit interleukin-1β. *Biochem Biophys Res Commun* **150**:1237-1243

Morimoto S, Nabata T, Koh E, Shiraishi T, Fukuo K, Imanaka S, Kitano S, Miyashita Y, Ogihara T (1991) Interleukin-6 stimulates proliferation of cultured vascular smooth muscle cells independently of interleukin-1 beta. *J Cardiovasc Pharmacol* **17**Suppl 2:S117-118

Morrisey JH (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. **117**:307-

Morzycki W, Issekutz AC (1991) Tumour necrosis factor-alpha but not interleukin-1 induces polymorphonuclear leukocyte migration through fibroblast layers by a fibroblast-dependent mechanism. *Immunol* **74**:107-113

Mosley B, Dower S, Gillis S, Cosman D (1987a) Determination of the minimum polypeptide lengths of the functionally active sites of human interleukins-1 α and β. *Proc Natl Acad Sci USA* **84**:4572-4576

Mosley B, Urdal D, Prickett K, Larsen D, Cosman D, Conlon P, Gillis S, Dower SK (1987b) The interleukin 1 receptor binds the human interleukin-1α precursor but not the interleukin-1β precursor. *J Biol Chem* **262**:2941-2944

Mulkins MA, Allison AC (1987) Recombinant human interleukin-1 inhibits the induction by dexamethasone of alkaline phosphatase activity in murine capillary endothelial cells. *J Cell Physiol [HNB]* **133**:539-545

Mulligan MS, Ward PA (1992) Immune complex-induced lung and dermal vascular injury. Differing requirements for tumor necrosis factor-α and IL-1. *J Immunol* **149**:331-339

Mullis KB, Faloona FA (1987) *Methods in Enzymology* **155**:335-350

Mundy GR (1991) Inflammatory mediators and the destruction of bone. *J Periodont Res* **26**:213-217

Munoz E, Zubiaga A, Sims JE, Huber BT (1991) IL-1 signal transduction pathways. I. Two functional IL-1 receptors are expressed in T cells. *J Immunol* **146**:136--143

Munoz E, Zubaiga AM, Huber BT (1992a) Interleukin-1 induces c-fos and c-jun gene expression in T-helper type II cells through different signal transmission pathways. *Eur J Immunol* **22**:2101-2106

Munoz E, Zubiaga A, Huang C-K, Huber BT (1992b) Interleukin-1 induces protein tyrosine kinase phosphorylation in T cells. *Eur J Immunol* **22**:1391-1396

Murphy PA, Chesney J, Wood WB Jr (1974) Further purification of rabbit leukocyte pyrogen. *J Lab Clin Med* **83**:310-322

Nagai K, Thogersen HC (1984) Generation of β-globin by sequence-specific proteolysis of a hybrid protein produced in E.coli. *Nature* **309**:810-812

Nagelkerken LM, van Breda Vriesman PJ (1986) Membrane-associated IL-1-like activity on rat dendritic cells. *J Imunol* **136**:2164-2170

Nagura H, Ohtani H, Masuda T, Kimura M, Nakamura S (1991) HLA-DR expression on M cells overlying Peyers patches is a common feature of human small intestine. *Acta Pathol Jpn* **41**:818-823

Naito K, Inaba K, Hirayama Y, Inaba-Miyama M, Sudo T, Muramatsu S (1989) Macrophage factors which enhance the mixed leukocyte reaction initiated by dendritic cells. *J Immunol* **142**:1834-1839

Naito Y, Fukata J, Tominaga T, Masui Y, Hirai Y,Murakami N, Tamai S, Mori K, Imamura H (1989) Adrenocorticotropin hormone releasing activities of interleukins in a homologous in vivo system. *Biochem Biophys Res Commun* **164**:1262-1267

Nakai S, Kawai K, Hirai Y, Tasaka K (1990) A mutant protein of human interleukin-1 beta with immunostimulatory but not pyrogenic potency. *Life Sci* **47**:1707-1714

Nanduri VB, Hulmes JD, Pan YC, Kilian PL, Stern AS (1991) The role of arginine residues in interleukin 1 receptor binding. *Biochim Biophys Acta* **1118**:25-35

Naylor MS, Malik ST, Stamp GW, Balkwill FR (1990) Expression of TNF by ovarian cancer cells in vivo. *European Federation of Immunological Societies10th Meeting, Edinburgh. Abstracts, 15-16*

Needleman SB, Wunch CD (1970) *J Mol Biol* **48**:443

Neefjes JJ, Ploegh HL (1992) Intracellular transport of MHC class II molecules. *Immunology Today* **13**:179-184

Neta R, Sztein MB, Oppenheim JJ, Gillis S, Douches SD (1987) The in vivo effects of interleukin 1. I. Bone marrow cells are induced to cycle after administration of interleukin1. *J Immunol* **139**:1861-1866

Nett MA, Ceretti DP, Berson DR, Seavitt J, Gilbert DJ, Jenkins NA, Copeland NG, Black RA, Chaplin DD (1992) Molecular cloning of the murine IL-1β converting enzyme cDNA. *J Immunol* **149**:3254-3259

Newton RC, Uhl J, Covington M, Back O (1988) The distribution and clearance of radiolabelled human interleukin-1 beta in mice. *Lymphokine Res* **7**:207-216

Newton RC (1987) Lack of a central role for calcium in the induction and release of human interleukin-1. *Biochem Biophys Res Commun* **147**:1027-1033

Nishida T, Hirato T, Nishino N, Mizuno K, Sekiguchi Y, Takano M, Kawai K, Nakai S, Hirai Y (1988a) Cloning of c-DNAs for rat interleukin-1 alpha and beta. *Prog in Leukocyte Biol* **8**:73-78

Nishida T, Takano M, Kawakami T, Nishino N, Nakai S, Hirai Y (1988b) The transcription of the interleukin 1 beta gene is induced with PMA and inhibited with dexamethasone in U937 cells. *Biochem Biophys Res Commun* **156**:269-274

Nishihara T, Ishihara Y, Noguchi T, Koga T (1989) Membrane IL-1 induces bone resorption in organ culture. *J Immunol* **143**:1881-1886

Nishimura Y, Bierer BE, Burakoff SJ (1988) Expression of CD5 regulates responsiveness to IL-1. *J Immunol* **141**:3438-3444

Numerof RP, Kotick AN, Dinarello CA, Mier JW (1990) Pro-interleukin-1β production by a subpopulation of human T cells, but not NK cells, in response to IL-2. *Cell Immunol* **130**:118-128

Nussenweig MC, Steinman RM, Unkeless JC, Witner MD, Gutchinov B, Cohn ZA (1981) Studies of the cell surface of mouse dendritic cells and other leukocytes. *J Exp Med* **154**:168-187

O'Neill LA, Bird TA, Gearing AJ, Saklatvala J (1990) Interleukin-1 signal transduction. Increased GTP binding and hydrolysis in membranes of a murine thymoma line (EL4). *J Biol Chem* **265**:3146-3152

Obal Jr F, Opp M, Cady AB, Johannsen L, Postlethwaite AE, Poppleton HM, Seyer JM, Kreuger JM (1990) Interleukin-1α and an interleukin-1β fragment are somnogenic. *Am J Physiol* **259**:R439-R466

Ochman H, Gerber AS, Hartl DL (1988) Genetic applications of an inverse polymerase chain reaction. *Genetics* **120**:621-623

Ohara O, Dorit RL, Gilbert W (1989) One-sided polymerase chain reaction: The amplification of cDNA. *Proc Natl Acad Sci USA* **86**:5673-5677

Ohlsson K, Bjork P, Bergenfeldt M, Hagemen R, Thompson RC (1990) Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. *Nature* **348**:550-552

Ohmori Y, Strassman G, Hamilton TA (1990) cAMP differentially regulates expression of mRNA encoding IL-1α and IL-1β in murine peritoneal macrophages. *J Immunol* **145**:3333-3339

Okamoto M, Sasano M, Goto M, Nishioka K, Aotsuka S, Nakamura K, Yokohari R, Miyamoto T (1991) Interleukin-1 beta release from human peripheral blood monocytes associated with phagocytosis of carbonyl-iron or erythrocytes. *Int J Immunopharmacol* **13**:45-50

Okuno Y, Takahashi T, Suzuki A, Fukumoto M, Nakamura K, Imura H (1991) Co-production of interleukin-1 and interleukin-6 in tumor cell lines elaborating colony-stimulating factors. *Jpn J Cancer Res* **82**:890-892

Okusawa S, Dinarello CA, Yancey KB, Endres S, Lawley TJ, Frank MM, Burke JF, Gelfand JA (1987) C5a induction of human interleukin-1. Synergistic effect with endotoxin or interferon-gamma. *J Immunol* **139**:2635-2640

Onozaki K, Tamatani T, Hashmoto T, Matsushima K (1987) Growth inhibition and augmentation of mouse myeloid leukemic cell line differentiation by interleukin 1. *Cancer Res* **47**:2397-2402

Oppenheim JJ, Koopman WJ, Wahl LM, Dougherty SF (1980) Prostaglandin E_2 rather than lymphocyte activating factor produced by activated human mononuclear cells stimulates increases in murine thymocyte cAMP. *Cell Immunol* **49**:64-73

Ostensen ME, Thiele DL, Lipsky PE (1989) Enhancement of human natural killer cell function by the combined effects of tumor necrosis factor-α or interleukin-1 and interferon-α or interleukin-2. *J Biol Response Mod* **8**:53-61

Otterness IG, Bliven ML,Eskra JD, Reinke M, Hanson DC (1988) The pharmocologic regulation of interleukin-1 production: the role of prostaglandins. *Cell Immunol* **114**:385-397

Palkama T, Matikainen S, Hurme M (1993) Tyrosine kinase activity is involved in the protein kinase C induced expression of interleukin-1 beta gene in monocytic cells. *FEBS Lett* **319**:100-104

Parker KP, Benjamin WR, Kaffka KL, Kilian PL (1989) Presence of IL-1 receptors on human and murine neutrophils. Relevance to IL-1-mediated effects in inflammation. *J Immunol* **142:**537-542

Pecceu F, Dousset P, Shire D, Cavrois E, Marchese E, Ferrara P, Kaghad M, Dumont X, Lupker J (1991) Human interleukin 1 beta fused to the human growth hormone signal peptide is N-glycosylated and secreted by chinese hamster ovary cells. *Gene* **97**:253-258

Pelletier JP, Faure MP, DiBattista JA, Wilhwlm S, Visco D, Martel-Pelletier J (1993) Coordinate synthesis of stromelysin, interleukin-1 and oncogene proteins in experimental osteoarthritis. An immunohistochemical study. *Am J Pathol* **142**:95-105

Peng R (1991) Distribution of S-100 protein-positive dendritic cells in transitional cell carcinoma of human bladder and its relatioon to clinical prognosis. *Chung-Hua I Hsueh Tsa Chih* **71**:579-580 [Abstract only]

Pepin M, Cannella D, Fontaine JJ, Pittet JC, Le Pape A (1992) Ovine mononuclear phagocytes in situ: Identification by monoclonal antibodies and involvement in experimental pyogranulomas. *J Leuk Biol* **51**:188-198

Peretti M, Mugridge KG, Becherucci C, Parente L (1991) Evidence that interleukin-1 and lipoxygenase metabolites mediate the lethal affect of complete Freund's adjuvant in adrenalectomised rats. *Lymphokine Cytokine Res* **10**:239-243

Perretti M, Flower RF (1993) Modulation of IL-1-induced neutrophil migration by dexamethasone and lipocortin 1. *J Immunol* **150**:992-999

Peters JH, Ruppert J, Fey-Ostermeier D, Freidrichs D, Xu H, Geiseler RKH (1992) Signals required for differentiating dendritic cells from human monocytes in vitro. *2nd Int symposium on dendritic cells in fundamental and clinical immunology, Amsterdam, The Netherlands. Abstracts*, p59

Phan SH, Gharaee-Kermani M, McGarry B, Kunkel SL, Wolber FW (1992) Regulation of rat pulmonary artery endothelial cell transforming growth factor-β production by IL-1β and TNF-α. *J Immunol* **149**:103-106

Picker LJ, Treer JR, Ferguson-Darnell B, Collins PA, Buck D, Terstappen LWMM (1993) Control of lymphocyte recirculation in man. I. Differential regulation of the peripheral lymph node homing receptor L-selectin on T cells during the virgin to memory cell transition. *J Immunol* **150**:1105-1121

Pistoia V, Cozzolino F, Rubartelli A, Torcia M, Roncella S, Ferrarini M (1986) In vitro production of interleukin 1 by normal and malignant human B lymphocytes. *J Immunol* **136**:1688-1692

Plebanski M, Elson CJ, Billington WD (1992) Dependency of interleukin-1 of primary human in vitro T cell responses to soluble antigens. *Eur J Immunol* **22**:2353-2358

Pober JS, LaPierre LA, Stolpen AH, Brock TA, Springer TA, Fiers W, Bevilacqua MP, Mendrick DL, Gimbrone MA (1987) Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin-1 species. *J Immunol* **138**:3319-3324

Poindexter K, Jerzy R, Gayle III RB (1991) Expression of interleukin 1 receptors on human preipheral T cells. *Nucl Acids Res* **19**:1899-1904

Poole S, Bird TA, Selkirk S, Gaines-Das RE, Choudry Y. Stephenson SL, Kenny AJ, Saklatvala J (1990) Fate of injected interleukin 1 in rats: sequestration and degradation in the kidney. *Cytokine* **2**:416-422

Porcelli S, Morita CT, Brenner MB (1992) CD1b restricts the response of human $CD4^-8^-$ T lymphocytes to a microbial antigen. *Nature* **360**:593-597

Postlethwaite AE, Lachmann LB, Mainardi CL, Kang AH (1983) Interleukin-1 stimulation of collagenase production by cultured fibroblasts. *J Exp Med* 157:801-806

Poutsiaka DD, Clark BD, Vannier E, Dinarello CA (1991) Production of interleukin-1 receptor antagonist and interleukin-1β by peripheral blood mononuclear cells is differentially regulated. *Blood* **78**:1275-1281

Presentini R, Perin F, Ancilli G, Bartalini M, Nucci D, Villa L, Boraschi D, Antoni G (1990) Radiolabelling of the biologically active peptide 163 -171 of human interleukin-1 beta. *Int J Rad Appl Instrum [A]* **41**:696-699

Priestle JP, Schar H-P, Grutter MG (1989) Crystallographic refinement of interleukin 1β at 2.0A resolution. *Proc Natl Acad Sci* **86**:9667-9671

Prowse PR, Baumann H (1988) Hepatocyte-stimulating factor, β_2-interferon and interleukin-1 enhance expression of the rat a_1-acid glycoprotein gene via a distal upstream regulatory region. *Mol Cell Biol* **8**:42-51

Pugh CW, MacPherson GG, Steer HW (1983) Characterisation of non-lymphoid cells derived from rat peripheral lymph. *J Exp Med* **157**:1758-1779

Pure E, Inaba K, Crowley MT, Tardelli L, Witmer-Pack MD, Rubertelli G, Fathman G, Steinman RM (1990) Antigen processing by epidermal Langerhans cells correlates with the level of biosynthesis of MHC class II molecules and expression of invariant chain. *J Exp Med* **172**:1459-1469

Quagliarello VG, Wispelwey B, Long WJ, Scheld WM (1991) Recombinant IL-1 induced meningitis and blood-brain barrier injury in the rat. Characterisation and comparison with TNF. *J Clin Invest* **87**:1306-1366

Qwarnstrom EE, Page RC, Gillis S, Dower DK (1988) Binding, internalisation and intracellular localisation of interleukin-1β in human diploid fibroblasts. *J Biol Chem* **263**:8261-8269

Racioppi L, Moscarella A, Ruggiero G, Manzo C, Ferrone S, Fontana S, Zappacosta S (1990) Inhibition by anti-HLA class II monoclonal antibodies of monoclonal antibody OKT-3-induced T cell proliferation. Studies at the mRNA level. *J Immunol* **145**:3635-3640

Ralph P, Nakoinz I, Samson-Johannes A, Fong S, Lowe D, Min H-Y, Lin L (1992) IL-10, T lymphocyte inhibitor of human blood cell production of IL-1 and Tumor necrosis factor. *J Immunol* **148**:808-814

Ray CA, Black RA, Kronheim SR, Greenstreet TA, Sleath PR, Salvesen GS, Pickup DJ (1992) Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1 beta converting enzyme. *Cell* **69**:597-604

Ray K, Thompson N, Kemmard N, Rollins P, Grenfell S, Witham S, Smithers N, Solari R (1992) Investigation of guanine-nucleotide-binding protein involvement and regulation of cyclic AMP metabolism in the interleukin 1 signal transduction. *Biochem J* **282**:59-67

Reid CD, Fryer PR, Clifford C, Kirk A, Tikerpae J, Knight SC (1990) Identification of hematopoetic progenitors of macrophage and dendritic Langerhans cells (DL-CFU) in human bone marrow and peripheral blood. *Blood* **76**:1139-1149

Reimers J, Wogensen LD, Welinder B, Hejnaes KR, Poulsen SS, Nilsson P, Nerup J (1991) The pharmacolkinetics, distribution and degradation of human recombinant interleukin 1 beta in normal rats. *Scand J Immunol* **34**:597-610

Reitamo S, Antilla HSI, Didierjean L, Saurat JH (1990) Immunohistochemical identification of interleukin-1 alpha and beta in human eccrine sweat-gland apparatus. *Br J Dermatol* **122**:315-323

Reyburn HT, Roy DR, Blacklaws BA, Sargan DR, McConnell I (1992) Expression of meadi-visna virus major core protein, p25: development of a sensitive p25 antigen detection assay. *J Virol Methods* **37**:305-320

Rhyne JA, Mizel SB, Taylor RG, Chedid M, McCall CE (1988) Characterisation of the human interleukin 1 receptor on human polymorphonuclear leukocytes. *Clin Immunol Immunopathol* **48**:354-361

Ridel PR, Jamet P, Robin Y, Bach MA (1986) Interleukin-1 released by blood monocyte-derived macrophages from patients with leprosy. *Infect Immun* **52**:303-308

Robert A, Olafsson AS, Lancaster C, Zhang WR (1991) Interleukin-1 is cryoprotective, antisecretory, stimulates PGE_2 synthesis by the stomach and retards gastric emptying. *Life Sci* **48**:123-134

Roberts B (1989) Nuclear location signal-mediated protein transport. *Biochim Biophys Acta* **1008**:263-280

Roberts NJ, Prill AH, Mann TN (1986) Interleukin 1 and interleukin 1 inhibitor production by human macrophages exposed to influenza virus or respiratory syncytial virus. *J Exp Med* **163**:511-519

Rodgers BC, Scott DM, Mundin J, Sissons JGP (1985) Monocyte-dreived inhibitor of interleukin 1 induced by human cytomegalovirus. *J Virol* **55**:527-532

Rollins P, Witham S, Ray K, Thompson N, Sadler H, Smithers N, Grenfell S, Solari R (1991) Modification of biological responses to interleukin-1 by agents that perturb singal transduction pathways. *Cytokine* **3**:42-53

Romani N, Lenz A, Glassl H, Stossel H, Stanzl U, Majdic O, Fritscg P, Schuler G (1989) Cultured human Langerhans cells resemble lymphoid dendritic cells in phenotype and function. *J Invest Dermatol* **93**:600-609

Rooney M, Symons J, Duff GW (1990) Interleukin 1 beta in synovial fluid is related to local disease activity in rheumatoid arthritis. *Rheumatol Int* **10**:217-219

Rosenwasser LJ, Webb AC, Clark BD, Irie S, Chang L, Dinarello CA, Gehrke L, Wolff SM, Rich A, Auron PE (1986) Expression of biologically active human interleukin 1 subpeptides by transfected simian COS cells. *Proc Natl Acad Sci USA* **83**:5243-5246

Rosoff PM (1990) IL-1 receptors: structure and signals. *Semin-Immunol* **2** :29-137

Rossi G, Heveker N, Thiele B, Gelderblom H, Steinbach F (1992a) Development of a Langerhans cell phenotype from peripheral blood monocytes. *Immunol Lett* **31**:189-198

Rossi G, Owsianowski M, Thiele B, Bogdanoff, Gollnick H (1992b) IL-6 and its high-affinity receptor during differentiation of monocytes into Langerhans' cells. *2nd Int symposium on dendritic cells in fundamental and clinical immunology, Amsterdam, The Netherlands. Abstracts,* p43

Rothwell NJ (1991) Functions and mechanisms of interleukin 1 in the brain. *Trends Pharmacol Sci* **12**:430-436

Roux-Lombard P, Modoux C, Dayer JM (1989) Production of interleukin-1 (IL-1) and a specific inhibitor during human monocyte-macrophage differentiation: influence of GM-CSF. *Cytokine* **1**:45-51

Rubartelli A, Bajetto A, Allavena G, Cozzolino F, Sitia R (1993) Post-translational regulation of interleukin 1β secretion. *Cytokine* **5**:117-124

Rubartelli A, Cozzolino F, Talio M, Sitia R (1990) A novel secretory pathway for interleukin 1b, a protein lacking a signal sequence. *EMBO J* **9**:1503-1510

Rubartelli A, Bajetto A, Allavena G, Cozzolino F, Sitia R (1993) Post-translational regulation of interleukin 1β secretion. *Cytokine* **5**:117-124

Ruco LP, Pisacane A, Pomponi D, Stoppacciaro A, Pescarmona E, Rendina EA, Santoni A, Boraschi D, Tagliabue A, Uccini S et al. (1990) Macrophages and interdigitating reticulum cells in normal human thymus and thymomas: immunoreactivity for inteleukin-1 aplha, interleukin-1 beta and tumour necrosis factor alpha. *Histopathol* **17**:291-299

Ruppert J, Peters JH (1991) Accessory function during monocyte/macrophage diferentiation: relation to interleukin-1 (IL-1β) production and release. *Eur J Cell Biol* **55**:352-361

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**:487-491

Saklatvala J, Curry VA, Sarsfield SJ (1983) Purification to homogeneity of pig leucoctye catabolin, a protein that causes cartilage resorption in vitro. *Biochem J* **215**:385-392

Saklatvala J, Bird T (1986) A common class of receptors for the two types of porcine interleukin-1 on articular cartilage. *Lymphokine Res* **5**:S99-104

Saklatvala J, Guesdon F (1991) Interleukin 1 signal transduction. *Agents Actions Suppl* **35**:35-40

Santiago-Schwarz F, Belilos E, Diamond B, Carsons SE (1992) TNF in combination with GM-CSF enhances the differentiation of neonatal cord blood stem cells into dendritic cells and macrophages. *J Leukoc Biol* **52**:274-281

Saperas ES, Yang H, Rivier C, Tache Y (1990) Central action of recombinant interleukin-1 to inhibit acid secretion in rats. *Gastroenterology* **99**:1599-1606

Sarno EN, Grau GE, Vieira LMM, Nery JA (1991) Serum levels of tumour necrosis factor-alpha and interleukin-1 beta during leprosy reactional states. *Clin Exp Immunol* **84**:103-108

Sato T (1987) Evidence of interleukin-1 like activity associated with bovine serum albumin. *J Clin Lab Immunol* **24**:39-43

Satsangi J, Wolstencroft RA, Cason J, Ainley CC, Dumonde DC, Thompson RP (1987) Interleukin 1 in Crohn's disease. *Clin Exp Immunol* **67**:594-605

Saurat JH, Schifferli J, Steiger G, Dayer JM, Diderjean L (1991) Anti-interleukin-1 alpha autoantibodies in humans: characterisation, isotype distribution, and receptor-binding inhibition--higher frequency in Schnitzler's syndrome (urticaria and macroglobulinemia). *J Allergy Clin Immunol* **88**:244-256

Savage N, Puren AJ, Orencole SF, Ikejima T, Clark BD, Dinarello CA (1989) Studies on IL-1 receptors on D10S T-helper cells: demonstration of two molecularly and antigenically distinct IL-1 binding proteins. *Cytokine* **1**:23-35

Sawada H, Kan M, McKeehan WL (1990) Opposite effects of monokines (interleukin-1 and tumour necrosis factor) on proliferation and heparin binding (fibroblast) growth factor binding to human aortic endothelial and smooth muscle cells. *In Vitro Cell Dev Biol* **26**:213-216

Scales WE, Chensue SW, Otterness I, Kunkel SL (1989) Regulation of monokine gene expression: prostaglandin E_2 suppresses tumor necrosis factor but not interleukin-1α or β-mRNA and cell-associated bioactivity. *J Leuko Biol* **45**:416-421

Scapigliati G, Ghiara P, Bartalini M, Tagliabue A, Boraschi D (1989) Differential binding of IL-1 alpha and IL-1 beta to receptors on B and T cells. *FEBS Lett* **243**:394-398

Scatchard G (1949) The attractions of proteins for small molecules and ions. *Ann N Y Acad Sci* **51**:660-672

Schade UF, Engel R, Jacobs D (1991) The role of lipoxygenases in endotoxin-induced cytokine production. *Prog Clin Biol Res* **367**:73-82

Schalke BCG, Klinkert WEF, Wekerle H, Dwyer DS (1985) Enhanced activation of a T cell line specific for acetylcholine receptor (AChR) by using anti-AChR monoclonal antibodies plus receptors. *J Immunol* **134**:3643-3648

Scheibenbogen C, Andreesen R (1991) Developmental regulation of the cytokine repertoire in human macrophages: IL-1, IL-6, TNF-alpha, and M-CSF. *J Leukoc Biol* **50**:35-42

Schiller B, Ziegler-Heitbrock HW, Meyer N, Schmidt B, Blumstein M (1991) Monocyte phenotype and interleukin-1 production in patients undergoing haemodialysis. *Nephron* **59**:573-579

Schindler R, Ghezzi P, Dinarello CA (1990a) IL-1 induces IL-1. IV. IFN-gamma suppresses IL-1 but not lipopolysaccharide-induced transcription of IL-1. *J Immunol* **144**:2216-2222

Schindler R, Gelfand JA, Dinarello CA (1990b) Recombinant C5a stimulates transcription rather than translation of interleukin-1 (IL-1) and tumor necrosis factor: translational signal provided by lipopolysaccharide or IL-1 itself. *Blood* **76**:1631-1638

Schindler R, Lonnemann G, Sheldon S, Koch KM, Dinarello CA (1990c) Transcription, not synthesis, of interleukin-1 and tumour necrosis factor by complement. *Kidney Int* **37**:85-93

Schindler R, Clark BD, Dinarello CA (1990d) Dissociation between interleukin-1 beta mRNA and protein systhesis in human peripheral blood mononuclear cells. *J Biol Chem* **265**:10232-10237

Schmidt JA, Bomford R (1991) The processing of interleukin-1 beta studied with antibodies raised against synthetic peptides from the precursor N-terminal region. *Cytokine* **3**:240-245

Schnyder J, Payne T, Dinarello CA (1987) Human monocyte or recombinant interleukin 1's are specific for the secretion of a metalloproteinase from chondrocytes. *J Immunol* **138**:496-503

Schuler G, Steinman RM (1985) Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *JExp Med* **161**:526-546

Schwarz T, Urbanska A, Gschnait F, Luger TA (1987) UV-irradiated epidermal cells produce a specific inhibitor of interleukin 1 activity. *J Immunol* **138**:1457-1463

Seckinger P, Lowenthal JW, Williamson K, Dower JM, MacDonald HR (1987) A urine inhibitor of interleukin 1 acitivity that blocks ligand binding. *J Immunol* **139**:1546-1549

Seow H-F, Rothel JS, David M-J, Wood PR (1991) Nucloetide sequence of ovine macrophage interleukin-1 beta cDNA. *DNA Sequence* **1**:423-426

Sertl K, Takemura T, Tschachler E, Ferrans VJ, Kaliner MA, Shevach EM (1986) Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma, and visceral pleura. *J Exp Med* **163**:436-451

Shalaby MR, Waage A, Aarden L, Espevik T (1989) Endotoxin, tumor necrosis factor-alpha and interleukin 1 induce interleukin 6 production in vivo. *Clin Immunol Immunopathol* **53**:488-498

Shamoto M, Hosokawa S, Shinzato M, Keneko C (1992) Comparison of Langerhans' cells and interdigitating reticulum cells. *2nd Int symposium on dendritic cells in fundamental and clinical immunology, Amsterdam, The Netherlands. Abstracts*, p68

Sharief MK, Ciardi M, Thompson EJ (1992) Blood-brain barrier damage in patients with bacterial meningitis: Association with TNFα but not IL-1β. *J Infect Dis* **166**:350-358

Shelly JA, Laborde AL (1992) Interleukin-1 binding, internalisation and processing in a murine osteoblastic cell line. *Eur Cytokine Netw* **3**:469-475

Shieh J-H, Gordon MS, Peterson RHF, Jakubowski AA, Gabrilove JL, Moore MAS (1990) Modulation of cytokine receptors and superoxide production in neutrophils treated with IL-1 in vitro and in vivo. *Blood* **76** (Suppl):165a

Shieh J-H, Peterson RH, Moore MA (1991) Granulocyte colony-stimulating factor modulation of cytokine receptors on murine bone marrow cells. In vivo and in vitro studies. *J Immunol* **147**:2984-2990

Shimozato T, Iwata M, Kawada H, Tamura N (1990) Human immunoglobulin preparation for intravenous use induces elevation of cellular cyclic adenosine 3':5'-monophosphate levels, resulting in suppression of tumour necrosis factor alpha and interleukin-1 production

Shinmei M, Masuda K, Kikuchi T, Shimomura Y (1989) The role of cytokines in chondrocyte mediated cartilage degradation. *J Rheumatol Suppl* **18**:32-34

Shirakawa F, Tanaka Y, Ota T, Suzuki H, Eto S, Yamashita U (1987) Expression of interleukin 1 receptors on human peripheral T cells. *J Immunol* **138**:4243-4248

Shiroo M, Matsushima K (1990) Enhanced phosphorlyation of 65 and 74 Kda proteins by tumor necrosis factor and interleukin-1 in human peripheral blood mononuclear cells. *Cytokine* **2**:13-20

Simms HH, Gaither TA, Fries LF, Frank MM (1991) Monokines released during short-term Fc gamma receptor phagocytosis up-regulate polymorphonuclear leukocytes and monocyte phagocytic function. *J Immunol* **147**:265-272

Sims JE, March CJ, Cosman D, Widmer MB, MacDonald HR, McMahan CJ, Grubin CE, Wignall JM, Jackson JL, Call SM, Friend D, Alpert AR, Gillis S, Urdal DL, Dower SK (1988) cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. *Science* **241**:585-589

Sims JE, Acres RB, Grubin CE, McMahan CJ, Wignall JM, March CJ, Dower SK (1989) Cloning the interkeukin 1 receptor from human T cells. *Proc Natl Acad Sci USA* **86**:8946-8950

Sleath PR, Hendrickson RC, Kronheim SR, March CJ, Black RA (1990) Substrate specificity of the protease that processes human interleukin-1 beta. *J Biol Chem* **265**:14526-14528

Smith MF, Eilden D, Brewer MT, Eisenberg SP, Arend WP, Gutierrez-Hartmann (1993) Human IL-1 receptor antagonist promoter: Cell-type specific activity and identification of regulatory regions. *J Immunol* **149**:2000-2007

Solari R, Smithers N, Page K, Bolton E, Champion BR (1990b) Interleukin 1 responsiveness and receptor expression by murine TH1 and TH2 clones. *Cytokine* **2**:129-141

Solari R (1990a) Identification and distribution of two forms of the interleukin 1 receptor. *Cytokine* **2**:21-28

Spalding DM, Koopman WJ, Eldridge JH, McGhee Steinman RM (1983) Accessory cells in murine Peyer's patch. I. Identification and enrichment of a functional dendritic cell. *J Exp Med* **157**:1646-1659

Spalding DM, Griffin JA (1986) Different pathways of differentiation of Pre-B cell lines are induced by dendritic cells and T cells from different lymphoid tissues. *Cell* **44**:507-515

Spriggs MK, Lioubin PJ, Slack J, Dower SK, Jonas, Cosman D, Sims JE, Bauer j (1990) Induction of an interleukin-1 receptor (IL-1R) on monocytic cells. Evidence that the receptor is not encoded by a T cell-type IL-1R mRNA. *J Biol Chem* **265**:22499-22505

Spry CJF, Pflug AJ, Janossy G, Humphrey JH (1980) Large mononuclear (veiled) cells with 'Ia-like' membrane antigens in human afferent lymph. *Clin Exp Immunol* **39**:750-755

Stashenko P, Dewhirst FE, Rooney ML, Desjardins LA, Heeley JD (1987) Interleukin-1 beta is a potent inhibitor of bone formation in vitro. *J Bone Mineral Res* **2**:559-565

Steinman RM (1991) The dendritic cell system and its role in immunogenicity. *Ann Rev Immunol* **9**:271-296

Stockman BJ, Scahill TA, Ray M, Ulrich E-L, Strakalaitis NA, Brunner DP, Yem AW Deibel MR Jr (1992) Secondary structure and topology of interleukin-1 receptor antagonist protein determined by heteronuclear three-dimensional NMR spectroscopy. *Biochemistry* **31**:5237-5245

Stoeckle MY (1991) Post-transcriptional regulation of gro alpha, beta, gamma and IL-8 mRNAs by IL-1 beta. *Nucleic Acid Res* **19**:917-920

Stoppacciaro A, Bossu P, Ghiara P, Ruco LP, Censini S, Scapigliati G, Nuti S, Tagliabue A, Baroni CD, Boraschi D (1991) Binding of IL-1β to IL-1R Type II at single cell level. *J Immunol* **147**:1561-1566

Stosic-Grujicic S, Lukic ML (1992) Glucocorticoid-induced keratinocyte derived interleukin-1 receptor antagonist (s). *Immunology* **75**:293-298

Stossel H, Koch F, Kampgen E, Stoger P, Lenz A, Heufler C, Romani N, Schuler G (1990) Disappearance of certain acidic organelles (Endosomes and Langerhans cell granules) accompanies loss of antigen presenting capacity upon culture of epidermal Langerhans cells. *J Exp Med* **172**:1471-1482

Stuart PM, Zlotnik A, Woodward JG (1988) Induction of class I and class II MHC antigen expression on murine bone-marrow-derived macrophages by IL-4 (B cell stimulatory factor 1). *J Immunol* **140**:1542-1547

Subramanian N, Bray MA (1987) Interleukin 1 releases histamine from human basophils and mast cells in vitro. *J Immunol* **138**:271-275

Sundar SK, Cierpial MA, Kamaraju LS, Long S, Hsieh S, Lorenz C, Aaron M, Ritchie JC,Weiss JM (1991) Human immunodeficiency virus glycoprotein (gp120) infused into rat brain induces interleukin 1 to elevate pituitary-adrenal activity and decrease peripheral cellular immune responses. *Proc Natl Acad Sci* **88**:11246-11250

Suttles J, Giri JG, Mizel SB (1990) IL-1 secretion by macrophages. Enhancement of IL-1 secretion and processing by calcium ionophores. *J Immunol* **144**:175-182

Suzuki H, Kamimura J, Atabe T, Kashiwagi H (1990) Demonstration of neutralising autoantibodies against IL-1α in sera from patients with rheumatoid arthritis. *J Imunol* **145**:2140-2146

Suzuki H, Atabe J, Kamimura J, Kashiwagi H (1991) Anti-IL-1α auoantiboodies in patients with rheumatic diseases and in healthy subjects. *Clin Exp Immunol* **85**:407-412

Svenson M, Bagge Hansen M, Bendtzen K (1990) Distribution and characterisation of autoantibodies to interleukin-1 alpha in normal human antisera. *Scand J Immunol* **32**:695-701

Swain SL, Bradley LM, Croft M,Tokonogy S, Atkins G, Weinberg AD, Duncan DD, Hedrick SM, Dutton RW, Huston G (1991) Helper T-cell subsets: phenotype function and role of lymphokines in regulation of their development. *Immunol Rev* **123**:115-144

Swerlick RA, Garcia-Gonzalez E, Kubota Y, Xu YL, Lawley TJ (1991) Studies of the modulation of MHC antigen and cells adhesion molecule expression on human dermal microvascular endothelial cells. *J Invest Dermatol* **97**:190-196

Symons JA, Eastgate JA, Duff GW (1991a) A soluble binding protein specific for interleukin 1 beta is produced activated mononuclear cells. *Cytokine* **2**:190-198

Symons JA, Eastgate JA, Duff GW (1991b) Purification and characterisation of a novel soluble receptor for interleukin 1. *J Exp Med* **174**:1251-1254

Szakal AK, Kosko MH, Tew JG (1989) Microanatomy of lymphoid tissue during humoral immune responses: structure function relationships. *Ann Rev Immunol* **7**:91-109

Tabor S, Richardson CC (1987) DNA sequence analysis with a modified bacteriophage T7 polymerase. *Proc Natl Acad Sci USA* **84**:4767-4771

Tafuri A, Bowers WE, Handler ES, Appel, Lew R, Greiner D, Mordes JP, Rossini AA (1993) High stimulatory activity of dendritic cells from diabetes-prone BioBreeding/Worcester rats exposed to macrophage-derived factors. *J Clin Invest* **91**:2040-2048

Tagliabue A, Ghiara P, Boraschi D (1991) Non-inflammatory peptide fragments of IL-1 as safe new-generation adjuvants. *ResImmunology* **141**:563-568

Takao T, Mitchell WM, de Souza EB (1991) Interleukin-1 receptors in mouse kidney: identification, localisation and modulation by lipopolysaccharide treatment. *Endocrinology* **128**:2618-2624

Takaue Y, Kawano Y, Reading CL, Watanabe T, Abe T, Ninomiya T, Shimizu E, Ogura T, Kuroda Y, Yokobayashi A et al., (1990) Effects of recombinant human G-CSF, GM-CSF, IL-3 and IL-1 alpha on the growth of purified human peripheral blood progenitors. *Blood* **76**:330-335

Takeuchi M, Nagai S, Nakada H, Aung H, Satake N, Kaneshima H, Izumi T (1992) Characterisation of IL-1 inhibitory factor released from human alveolar macrophages as IL-1 receptor antagonist. *Clin Exp Immunol* **88**:181-187

Takii T, Akahoshi T, Kato K, Hatashi H, Marunouchi T, Onozaki K (1992) Interleukin-1 up-regulates transcription of its own receptor in a human fibroblast cell line TIG-1: role of endogenous PGE_2 and cAMP. *Eur J Immunol* **22**:1221-1227

Tanaka Y, Shirakawa F, Oda S, Eto S, Yamashita U (1989) Expression of IL-1 receptors on human peripheral B cells. *J Immunol* **142**:167-172

Tang A, Amagai M, Granger LG, Stanley JR, Udey MC (1993) Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. *Nature* **361**:82-85

Taylor DJ, Evanson JM, Wooley DE (1988) Comparative effects of of interleukin-1 and a phorbol ester on rheumatoid synovial cell fructose 2,6-bisphosphate content and prostaglandin E production. *Biochem Biophys Res Commun* **150**:349-354

te Velde AA, Huijbens RJ, Heije K, de Vries JE, Figdor CG (1990) Interleukin-4 (IL-4) inhibits secretion of IL-1 beta, tumor necrosis factor alpha and IL-6 by human monocytes. *Blood* **76**:1392-1397

Teodorescu M, McAfee M, Skosey JL, Wallman J, Shaw A, Hanly WC (1991) Covalent disuphide binding of human IL-1 beta to alpha 2-macroglobulin: inhibition by D-penicillamine. *Mol Immunol* **28**:323-331

Teunissen MBM, Wormmeester J, Krieg SR, Peters PJ, Vogels IMC, Kapsenberg ML, Bos JD (1990) Human epidermal Langerhans' cells undergo profound morphological changes during in vitro culture. *J Invest Dermatol* **94**:166-173

Thieme TR, Hefeneider SH, Wagner CR, Burger DR (1987) Recombinant murine and human IL-1α bind to human endothelkial cells with an equal affinity, but have an unequal ability to induce endothelial cell adherence of lymphocytes. *J Immunol* **139**:1173-1178

Thomas PS (1980) Hybridisation of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci* **77**:5201-5205

Thomas R, Davis LS, Lipsky PE (1993) Isolation and characterisation of human peripheral blood dendritic cells. *J Immunol* **150**:821-834

Thomson BM, Saklatvala J, Chambers TJ (1986) Osteoblasts mediate interleukin 1 stimulation of bone resorption by rat osteoclasts. *J Exp Med* **164**:104-112

Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Auronins J et al. (1992) A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. *Nature* **356**:768-774

Tobler I, Borbely A, Schwyzer M, Fontana A (1984) Interleukin-1 derived from astrocytes enhances slow wave activity in sleep EEG in the rat. *Eur J Pharmacol* **104**:191-192

Tocci MJ, Hutchinson NI, Cameron PM, Kirk KE, Norman DJ, Chin J, Rupp EA, Limjuco GA, Bonilla-Argudo VM, Schmidt JA (1987) Expression in Escherischia coli of fully active recombinant human IL-1 beta:comparison with native human IL-1 beta. *J Immunol* **138**:1109-1114

Todd S, Naylor SL (1991) Dinucleotide repeat polymorphism in the human interleukin 1 alpha gene (IL1α). *Nucl Acid Res* **19**:3756

Tovey MG, Content J, Gresser I, Gugenheim J, Blanchard B , Guymarho J, Poupart P, Gigou M, Shaw A, Fiers W (1988) Genes for IFN-beta-2 (Il-6), tumor necrosis factor, and IL-1 are expressed at high levels in the organs of normal individuals. *J Immunol* **141**:3106-3110

Tsai V, Bergroth V, Zvaifler NJ (1989) Dendritic cells in health and disease. *J Autoimmun* **2** Suppl:33-43

Tsujitani S, Kakeji Y, Watanabe A, Kohnoe S, Maehara Y, Sugimaghi K (1992) Infiltration of S-100 protein positive dendritic cells and peritoneal recurrence in advanced gastric cancer. *Int Surgery* **77**:238-241

Turner M, Chantry D, Buchan G, Barrett K, Feldmann M (1989) Regulation of expression of human IL-1 alpha and IL-1 beta genes. *J Immunol* **143**:3556-3561

Turner M, Chantry D, Katsikis P, Berger A, Brennan FM, Feldmann M (1991) Induction of the interleukin 1 receptor antagonist protein by transforming growth factor-β. *Eur J Immunol* **21**:1635-1639

Ucla C, Roux-Lombard P,Fey S, Dayer JM, Mach B (1990) Interferon gamma drastically modifies the regulation of interleukin 1 genes by endotoxin in U937 cells. *J Clin Invest* **85**:185-191

Uehara A, Gottshall PE, Dahl RR, Arimura A (1987) Stimulation of ACTH release by human interleukin-1 beta, but not by interleukin-1 alpha, in conscious freely-moving rats. *Biochim Biophys Res Commun* **146**:1286-1290

Uhl J, Newton RC, Giri JG, Sandlin G, Horuk R (1989) Identification of IL-1 receptors on human monocytes. *J Immunol* **142**:1576-1581

Ulich TR, Guo KZ, Irwin B, Remick DG, Davatelis GN (1990) Endotoxin-induced cytokine gene expression in vivo. II. Regulation of tumor necrosis factor and interleukin-1 alpha/beta expression and suppression. *Am J Pathol* **137**:1173-1185

Unemori EN, Hibbs MS, Amento EP (1991) Constitutive expression of a 92-kD gelatinase (type V collagenase) by rheumatoid synovial fibroblasts and its induction in normal human fibroblasts by inflammatory cytokines. *J Clin Invest* **88**:1656-1662

Urdal DL, Call SM, Jackson JL, Dower SK (1988) Affinity purification and chemical analysis of the interleukin-1 receptors. *J Biol Chem* **163**:2870-2877

Usui N, Mimnaugh EG, Sinha BK (1991) A role for the interleukin 1 receptor in the synergistic antitumor effects of human interleukin 1 alpha and etopside against melanoma cells. *Cancer Res* **51**:769-774

Uyemura K, Rea TH, Bloom BR, Modlin RL (1992) Cytokine patterns of immunologically mediated tissue damage. *J Immunol* **149**:1470-1475

Vakkila J, Sihova M, Hurme M (1990) Human peripheral blood-derived dendritic cells do not produce interleukin 1 alpha, interleukin 1 beta or interleukin 6. *Scand J Immunol* **31**:345-352

van Bruggen I, Price P, Robertson TA, Papadimitriou JM (1989) Morphological and functional changes during cytomegalovirus replication in murine macrophages. *J Leukoc Biol* **46**:508-520

van Damme J, van Beeuman J, Opdenakker G, Billian A (1988) A novel NH_2-terminal sequence - characterised monokine possessing neutrophil chemotactic, skin-reactive and granulocytosis-promoting activity. *J Exp Med* **167**:1364-1376

van de Rijn M, Lerch PG, Bronstein BR, Knowles RW, Bhan AK, Terhorst C (1984) Human cutaneous dendritic cells express two glycoproteins T6 and M241 which are biochemically identical to those found on cortical thymocytes. *Hum Immunol* **9**:201-210

van der Schoot E, Jansen P, Poorter M, Wester MR, von dem Borne AE, Aarden LA, van Oers RH (1989) Interleukin-6 and interleukin-1 production in acute leukemia with monocytoid differentiation. *Blood* **74**:2081-2087

van Voorhis WC, Hair LS, Steinman RM, Kaplan G (1982) Human dendritic cells. Enrichment and characterisation from peripheral blood. *J Exp Med* **155**:1172-1187

van Wilsem E, Breve J, van Hoogstraten I, Savelkoul H, Kraal G (1992) The regulation of T-cell cytokine production by dendritic cells. *2nd Int symposium on dendritic cells in fundamental and clinical immunology, Amsterdam, The Netherlands. Abstracts,* p23

Vannier E, Dinarello CA (1991) Interleukin-1 induces interleukin-1. VI. Histamine via the H-2 receptor enhances IL-1 induced IL-1 gene expression and synthesis.

Vannier E, Miller LC, Dinarello CA (1992) Coordinated anti-inflammatory effects of interleukin-4: interleukin-4 suppresses interleukin-1 production but upregulates gene expression and synthesis of interleukin-1 receptor antagonist. *Proc Natl Acad Sci* **89**:4076-4080

Veerapandian B, Gilliland GL, Raag R, Svensson AL, Masui Y, Hirai Y, Poulos TL (1992) Functional implications of interleukin-1 beta based on the three-dimensional structure. *Proteins* **12**:10-23

Waalen K, Duff GW, Forre O, Dickens E, Kvarnes L, Nuki G (1986) Interleukin 1 activity produced by human rheumatoid and normal dendritic cells. *Scand J Immunol* **23**:365-371

Wagner CR, Vetto RM, Burger DR (1985) Expression of I-region-associated antigen (Ia) and interleukin-1 by subcultured human epithelial cells. *Cell Immunol* **93**:91-104

Wainberg MA, Numazaki K, Destephano L, Wong I, Goldman H (1988) Infection of human thymic epithelial cells by human cytomegalovirus and other viruses: effect on secretion of interleukin 1-like activity. *Clin Exp Immunol* **72**:415-421

Warren JS (1991) Intrapulmonary interleukin 1 mediates acute immune complex alveolitis in the rat. *Biochem Biophys Res Commun* **175**:604-610

Watson S Bullock W, Nelson K, Schauf V, Gelber R, Jacobsen R (1984) Interleukin 1 production by peripheral blood mononuclear cells from leprosy patients. *Infect Immun* **45**:787-789

Weaver CT, Unanue ER (1986) T cell induction of membrane IL-1 on macrophages. *J Immunol* **137**:3868-3873

Weitzmann MN, Savage N (1992) Nuclear internalisation and DNA binding activities of interleukin-1, interleukin-1 receptor and interleukin-1 receptor complexes. *Biochem Biophys Res Commun* **187**:1166-1171

Wensink PC, Finnegan DJ, Donelson JE, Hogness DS (1974) A system for mapping DNA sequences in the chromosomes of Drosophila melanogaster. *Cell* **3**:315-325

Westacott CI, Wicher JT, Barnes IC, Thompson D, Swan AJ, Dieppe PA (1990) Synovial fluid concentrations of five different cytokines in rheumatic diseases. *Ann Rheum Dis* **49**:676-681

Wijelath ES, Kardasz AM, Drummond R, Watson J (1988) Interleukin-one induced inositol phospholipid breakdown in murine macrophages: possible mechanism of receptor activation. *Biochem Biophys Res Commun* **152**:392-397

Williams IR, Unanue ER (1991) Characterisation of accessory cells costimulation of Th1 cytokine synthesis. *J Immunol* **147**:3752-3760

Wilson AB, Harris JM, Coombs RR (1988) Interleukin-2 induced production of interferon-gamma by resting human T cells and large granular lymphocytes: requirement for accessory factors including interleukin-1. *Cell Immunol* **15**:130-142

Witmer-Pack MD, Valinsky J, Oliver W, Steinman RM (1988) Quantitation of surface antigens on cultured murine epidermal Langerhans' cells. Rapid and selective increase in the level of surface MHC products. *J Invest Dermatol* **90**:387-394

Wolff SM (1973) Biological effects of bacterial endotoxins in man. *J Infect Dis* **128**Suppl:S259-264

Wolfson AJ, Kanaoka M, Lau FT, Ringe D (1991) Insertion of an elastese-binding loop into interleukin-1 beta. *Protein Eng* **4**:313-317

Wong HL, Costa GL, Lotze MT, Wahl SM (1993) Interleukin (IL) 4 differentially regulates monocyte IL-1 family gene expression and synthesis in vitro and in vivo. *J Exp Med* **177**:775-781

Wood P (1993) CSIRO, Melbourne, Australia. Personal communication.

Xing Z, Jordana M, Gauldie J (1992) IL-1 beta and IL-6 gene expression in alveolar macrophages: modulation by extracellular matrices. *Am J Physiol* **262**:L600-605

Yamamura M, Nishiya K, Ota Z (1990) Increased ability of peripheral blood B cells from patients with rheumatoid arthritis to produce interleukin 1 in vitro. *Acta Med Okayama* **44**:301-308

Yamaoshi M, Ohue M, Kawashima H, Kotani H, Iida M, Kawata S, Yamada M (1990) A human IL-1α derivative which lacks prostaglandid E_2 inducing activity and inhibits the activity of IL-1 through receptor competeition. *Lymphokine Res* **9**:405-413

Yamato K, el-Hajjaoui Z, Koeffler HP (1989) Regulation of levels of IL-1 in human fibroblasts. *J Cell Physiol* **139**:610-616

Yamato K, el-Hajjaoui Z, Simon K, Koeffler HP (1990) Modulation of interleukin-1 beta RNA in monocytic cells infected with human immunodeficiency virus-1. *J Clin Invest* **86**:1109-1114

Yanisch Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors.*Gene* **33**:103- 119

Yanofsky SD, Zurawski G (1990) Identification of key residues in the amino terminal third of human interleukin-1α. *J Biol Chem* **26**:13000-13006

Yap SH, Moshage HJ, Hazenberg BP, Roelofs HM, Bijzet J, Limberg PC, Aarden LA, van Rijswijk MH (1991) Tumor necrosis factor (TNF) inhibits interleukin (IL)-1 and/or IL-6 stimulated syntheisi of C-reactive

protein (CRP) and serum amyloid A (SAA) in primary cultures of human hepatocytes. *Biochim Biophys Acta* **1091**:405-408

Yem AW, Epps DE, Matthews WR, Guido DM, Richard KA, Staite ND, Deibel MR Jr. (1992) Site-specific chemical modification of interleukin -1β by acrylodan at cysteine 8 and Lys 103. *J Biol Chem* **267**:3122-3128

Yong K, Cohen H, Khwaja A, Jones HM, Linch DC (1991) Lack of effect of granulocyte-macrophage and granulocyte colony-stimulating factors on cultured human endothelial cells. *Blood* **77**:1675-1680

Yong KL, Linch DC (1993) Granulocyte-macrophage-colony-dtimulating factor differentially regulates neutrophil migration across IL-1-activated and non-activated human endothelium. *J Immunol* **150**:2449-2456

Zhang G, Duff GW (1992) Interleukin-1β gene regulation. *British Soc. Immunology, Spring meeting, Sheffield University* :46(3.39)

Zhang JD, Cousens LS, Barr PJ, Sprang SR (1991) Three-dimensional structure of human basic fibroblast growth factor, a structural homolog of interleukin 1 beta. *Proc Natl Acad Sci USA* **88**:3446-3450

Zhu X, Komiya H, Chirino A, Faham S, Fox GM, Arakawa T, Hsu BT, Rees DC (1991) Three-dimensional structures of acidic and basic fibroblast factors. *Science* **251**:90-93

Ziegler-Heitbrock HWL, Strobel M, Kieper D, Fingerle G, Schlunck T, Petersmann I, Ellwart J, Blumstein M, Haas JG (1992) Differential expression of cytokines in human blood monocyte subpopulations. *Blood* **79**:503-511

Zoja C, Wang JM, Betton S, Sironi M, Renzi D, Chiaffarino F, Abboud HE, Van Damme J, Mantovani A, Remuzzi G et al (1991) Interleukin-1 beta and tumor necrosis factor-alpha induce gene expression and production of leukocyte chemotactic factors, colony-stimulating factors and interleukin-6 in human mesangial cells. *Am J Path* **138**:991-1003

Zucali J, Moreb J, Weiner R (1991) Effect of IL-1 and TNF-alpha on early progenitor cells: implications for bone marrow purging. *Bone Marrow Transplant* **7**Suppl:140

Zuckerman SH, Evans GF, Butler LD (1991) Endotoxin tolerance: independent regulation of interleukin-1 and tumor necrosis factor expression. *Infect Immun* **59**:2774-2780

PUBLICATIONS

Publications arising from work presented in this thesis are as follows:

1. Fiskerstrand C, Sargan D (1990) Nucleotide sequence of ovine interleukin-1 beta. *Nucl Acids Res* **18**:1765

2. Fiskerstrand CE, Roy DJ, Green I, Sargan DR (1992) Cloning, expression and characterisation of ovine interleukins-1α and β. *Cytokine* **4**:418-428

3. Fiskerstrand C, Hopkins J, Green I, Sargan D. Upregulation of expression of receptors for interleukin-1 and tumour necrosis factor on ovine afferent lymph dendritic cells after secondary antigen challenge. *Manuscript in preparation.*

4. Green IR, Fiskerstrand C, Bertoni G, Roy DJ, Peterhans E, Sargan D (1993) Expression and characterisation of bioactive recombinant ovine TNFα: Some species specificity in cytotoxic responses to TNF. Cytokine **5**:

Nucleotide sequence of ovine interleukin-1 beta

Carolyn Fiskerstrand and David Sargan
Department of Veterinary Pathology, University of Edinburgh, Edinburgh EH9 1QH, UK

Submitted October 25, 1990

EMBL accession no. X54796

The interleukins have been the subject of intense research interest because of their central role in the coordination of the processes of immune cell growth and differentiation, inflammation and responses to antigenic stimulation. Central to an understanding of these processes has been the cloning of the genes encoding these mediators. We have used PCR to obtain 3 overlapping clones which together constitute the ovine IL-1β c-DNA.

When ovine alveolar macrophages were stimulated with 100 ng/ml lipopolysaccharide from *Salmonella abortus equi* for 4 hours they produced IL-1 like activity as measured by a thymocyte co-proliferation assay. Total RNA was extracted from these cells and enriched for polyA$^+$ RNA by oligo dT-cellulose chromatography. cDNA synthesised from this RNA by the method of Gubler and Hoffman (1) was hybridised to primers chosen from sequences conserved in bovine (2) and human (3) IL-1β genes (nucleotide numbers 171−190 and antisense of 690−710 of the bovine gene). The cDNA was amplified by PCR to produce a central segment of the ovine gene. This segment was sequenced and from this sequence further primers were chosen for amplifying 5′ and 3′ regions of the gene. A similar cDNA population was tailed with dATP using terminal transferase, (so that both sense and antisense strands ends would have polyA tracts) and IL-1β molecules in it amplified using anchored PCR with a new primer (antisense of nucleotide numbers 297−314 of the ovine sequence) and oligo dT to generate the 5′ end. PCR with a further pair of primers (nucleotides 457−474 of the ovine sequence, and antisense of 995−1011 from the bovine 3′ untranslated region) was used to generate the 3′ end. (Anchored PCR using oligo dT did not give a full length product at the 3′ end). The 3 PCR products were cloned into pTZ18R expression vectors and several clones of each type sequenced using the dideoxy chain termination method with T7 DNA polymerase (Sequenase II, USB Ltd). The clones obtained encode an ovine IL-1β sequence consisting of 32 bp of 5′ leader sequence, an 801 bp coding sequence and 145 bp of 3′ untranslated sequence. Comparison of this sequence with bovine and human IL-1β shows similarities of 95.3% and 76.1% respectively at the nucleic acid level, or 84.9% and 57.5% at the protein level.

ACKNOWLEDGEMENTS

This work was supported by AFRC grant number AG 15/329, and in part by a Wellcome Trust Project Development Award.

REFERENCES

1. Gubler,U. and Hoffman,B.J. (1983) *Gene* **25**, 263−269.
2. Maliszewski,C.R., Baker,P.E., Schoenberg,M.A., Davis,B.S., Cosman,D., Gillis,S. and Cerretti,D.P. (1988) *Molec. Immunol.* **25**, 429−437.
3. March,C.J., Mosley,B., Larsen,A., Cerretti,D.P., Braedt,G., Price,V., Gillis,S., Henney,C.S., Kronheim,S.R., Grabstein,K., Conlon,P.J., Hopp,T.P. and Cosman,D. (1985) *Nature* **315**, 641−647.

```
                                                    GAACCTTCATTGCCCAGGTTTCTGAAACAGCC   -1

ATGGCAACCGTACCTGAACCCATCAATGAAGTGATGGCTTGCTACAGTGATGAGAATGAG   60
M  A  T  V  P  E  P  I  N  E  V  M  A  C  Y  S  D  E  N  E    20

CTGTTATTTGAGGTTGATGGCCCTAAACAGATGAAGAGCTGCACCCAACACCTGGACCTC  120
L  L  F  E  V  D  G  P  K  Q  M  K  S  C  T  Q  H  L  D  L    40

GGCTCCATGGGAGATGGAAACATCCAGCTGCAGATTTCTCACAAGCTCTACAACAAAAGC  180
G  S  M  G  D  G  N  I  Q  L  Q  I  S  H  K  L  Y  N  K  S    60

TTCAGTCAGGCAGTGTCGGTCATCGTGGCCATGGAGAAGCTGAGGAGCCGTGCCTACGAA  240
F  R  Q  A  V  S  V  I  V  A  M  E  K  L  R  S  R  A  Y  E    80

CATGTCTTCCGTGATGATGACCTGAGGAGCATCCTTTCATTCATCTTCGAAGAAGAGCCT  300
H  V  F  R  D  D  D  L  R  S  I  L  S  F  I  F  E  E  E  P   100

GTCATCTTCGAAACATCCTCCGATGAGCTTCTGTGTGATGCAGCCGTGCAGTCAGTAAAA  360
V  I  F  E  T  S  S  D  E  L  L  C  D  A  A  V  Q  S  V  K   120

TGCAAACTCCAGGACAGAGAGCAAAAATCCCTGGTGCTGGATAGCCCATGTGTGCTGAAG  420
C  K  L  Q  D  R  E  Q  K  S  L  V  L  D  S  P  C  V  L  K   140

GCTCTCCACCTCCTCTCACAGGAAATGAGCCGAGAAGTGGTGTTCTGCATGAGCTTCGTA  480
A  L  H  L  L  S  Q  E  M  S  R  E  V  V  F  C  M  S  F  V   160

CAAGGAGAGGAAAGAGACAACAAGATTCCTGTGGCCTTGGGTATCAGGGACAAGAATCTA  540
Q  G  E  E  R  D  N  K  I  P  V  A  L  G  I  R  D  K  N  L   180

TACCTGTCTTGTGTGAAAAAAGGTGATACACCGACCCTGCAGCTGGAGGAAGTAGACCCC  600
Y  L  S  C  V  K  K  G  D  T  P  T  L  Q  L  E  E  V  D  P   200

AAAGTCTACCCCAAGAGGAATATGGAAAAGCGATTCGTCTTCTACAAGACAGAAATCAAG  660
K  V  Y  P  K  R  N  M  E  K  R  F  V  F  Y  K  T  E  I  K   220

AACACAGTTGAATTTGAGTCTGTCCTGTACCCTAACTGGTACATCAGCACTTCTCAAATC  720
N  T  V  E  F  E  S  V  L  Y  P  N  W  Y  I  S  T  S  Q  I   240

GAAGAAAAGCCCGTCTTCCTGGGACGTTTTAGAGGTGGCCAGGATATAACTGACTTCAGA  780
E  E  K  P  V  F  L  G  R  F  R  G  G  Q  D  I  T  D  F  R   260

ATGGAAACCCTCTCTCCCTAAAGAAGCCATACGCAGGGGTCCACGTGGGCTGAATAACCCCGAGGAC  847
M  E  T  L  S  P  *                                          266

TGGCAGAAGGGAAGGGAAGAACACCAGCCGCAGCCTGAACCTCACTGTTGTGTGATCCATGCCCAACTGCC  918

TCCCCTGTATTCGTGCTGAGACGCTCTC                                          946
```


Nucleotide sequence of the tamarillo mosaic virus coat protein gene

Robin M.Eagles[1, 2], Richard C.Gardner[2] and Richard L.S.Forster[1]
[1]DSIR Plant Protection, Private Bag, Auckland and [2]Department of Cellular and Molecular Biology, University of Auckland, Auckland, New Zealand

Submitted October 25, 1990 EMBL accession no. X54804

Tamarillo mosaic virus (TaMV), a member of the potyvirus group, causes yield loss and fruit blemishing in tamarillo (*Cyphomandra betacea* Sendt.) (1). Potyvirus genomes contain approximately 10 kb of a single stranded RNA and are translated as a polyprotein that is subsequently proteolytically cleaved.

A cDNA clone covering the 3′ terminus of TaMV (strain R) has been isolated and sequenced; part of this sequence is shown in the figure below. N-terminal sequencing of the first 10 amino acids of purified virus coat protein identified the beginning of the coat protein gene in the cDNA sequence (sequenced amino acids are underlined). The amino acid residues immediately upstream of the coat protein gene are homologous to proteolytic cleavage sites preceding the coat protein genes of other potyviruses (2, 3). T_7 RNA transcripts were synthesized from a cDNA clone containing an in-frame AUG initiation codon immediately 5′ of the coat protein gene. An *in vitro* translation product synthesized from this RNA comigrated with the native coat protein and was immunoprecipitated by antibodies raised against purified virus (data not shown).

The TaMV coat protein shows 55–66% amino acid identity to the coat proteins of eleven distinct members of the potyvirus group. This level of homology suggests that TaMV is an independent member of the potyvirus group rather than a strain of an existing member (4). The TaMV 3′ untranslated sequence shows less than 50% homology with other potyviruses, providing further support for this conclusion (5).

ACKNOWLEDGEMENTS

This work was supported by the Agricultural Marketing Research and Development Trust and the New Zealand Tamarillo Growers' Association.

REFERENCES

1. Mossop,D.W. (1977) *N.Z. J. Agr. Res.* **20**, 535–541.
2. Dougherty,W.G., Cary,S.M. and Parks,T.D. (1989) *Virology* **171**, 356–364.
3. Domier,L.L., Franklin,K.M., Shahabuddin,M., Hellmann,G.M., Overmeyer,J.H., Hiremath,S.T., Siaw,M.F.E., Lomonossoff,G.P., Shaw,J.G. and Rhoads,R.E. (1986) *Nucl. Acids Res.* **14**, 5417–5430.
4. Shukla,D.D. and Ward,C.W. (1989) *Adv. Virus Res.* **36**, 273–314.
5. Frenkel,M.J., Ward,C.W. and Shukla,D.D. (1989) *J. Gen. Virol.* **70**, 2775–2783.

```
   1  GTAGATGAAGAGGATGACATAGTCTATTTCCAGGCTGGAACCCTTGATGCAGGTGAAGCGACAGCACAAAAAGCAGAGGGGAAGAAGAAGGAAGGAGAGG  100
      V  D  E  E  D  D  I  V  Y  F  Q  A  G  T  L  D  A  G  E  A  T  A  Q  K  A  E  G  K  K  K  E  G  E  V
 101  TTTCAAGCGGGAAAGCCGTCGTTGTAAAAGATAAGGATGTCGATTTAGGTACTGCTGGGACACATTCAGTACCACGCTTAAAGTCAATGACATCAAAGCT  200
        S  S  G  K  A  V  V  V  K  D  K  D  V  D  L  G  T  A  G  T  H  S  V  P  R  L  K  S  M  T  S  K  L
 201  CACACTGCCAATGCTCAAAGGTAAAAGTCGTTGTAACTTAGATCACTTGCTATCCTATAAACAAACAGTCGATTTATCAAATGCTAGAGCCACCCACGAA  300
        T  L  P  M  L  K  G  K  S  R  C  N  L  D  H  L  L  S  Y  K  Q  T  V  D  L  S  N  A  R  A  T  H  E
 301  CAATTTCAAAATTGGTATGATGGTGTGATGGCTAGTTATGAGCTGGAAGAATCAAGCATGGAAATCATCCTCAATGGTTTTATGGTATGGTGCATTGAAA  400
      Q  F  Q  N  W  Y  D  G  V  M  A  S  Y  E  L  E  E  S  S  M  E  I  I  L  N  G  F  M  V  W  C  I  E  N
 401  ATGGGACATCTCCTGACATAAATGGAGTCTGGACCATGATGGACGATGAAGAACAGATATCGTACCCACTTAAACCCATGCTTGATCATGCAAAGCCTTC  500
        G  T  S  P  D  I  N  G  V  W  T  M  M  D  D  E  E  Q  I  S  Y  P  L  K  P  M  L  D  H  A  K  P  S
 501  TTTAAGGCAAATAATGAGGCATTTCAGTGCGCTCGCAGAGGCGTATATTGAGATGAGAAGTCGTGAGAAGCCATACATGCCCAGGTATGGTTTACAACGC  600
        L  R  Q  I  M  R  H  F  S  A  L  A  E  A  Y  I  E  M  R  S  R  E  K  P  Y  M  P  R  Y  G  L  Q  R
 601  AATTTGAGAGATCAAAGTTTGGCAAGGTATGCATTTGATTTCTATGAGATCACTGCAACCACTCCGGTCAGAGCCAAGGAGGCGCATTTGCAAATGAAGG  700
      N  L  R  D  Q  S  L  A  R  Y  A  F  D  F  Y  E  I  T  A  T  T  P  V  R  A  K  E  A  H  L  Q  M  K  A
 701  CAGCTGCGCTGAAGAATTCGAACACTAACATGTTTGGACTGGACGGAAATGTCACAACTTCGGAAGAGGACACAGAAAGGCACACAGCAACGGATGTTAA  800
        A  A  L  K  N  S  N  T  N  M  F  G  L  D  G  N  V  T  T  S  E  E  D  T  E  R  H  T  A  T  D  V  N
 801  TCGCAACATGCATCACCTTCTGGGCGTGAAGGGGGTGTAACCGAAGTGCCGTACTATTATTATATATAAGAGTTCCTGTTAGTATCCTTGTCCTATTATT  900
        R  N  M  H  H  L  L  G  V  K  G  V
 901  AGAATACGTTTATCTTTCAACAAGTATGATATTCGTGCTCTCACCTCGCAATAGTGAGTTTGAGTGGGAAATAAGTACTAGTATTCTATGGCCTTACTTG 1000
1001  TTATATAGACCCACCACGTAGTGAGATTTTATCTCGGTGAATGGTTTTATGTTTCAACTGTCAGGG (A)27                              1066
```

Original Contributions

CLONING, EXPRESSION AND CHARACTERIZATION OF OVINE INTERLEUKINS 1α AND β

C.E. Fiskerstrand,* D.J. Roy, I. Green, D.R. Sargan

Ovine interleukin 1α (IL-1α) c-DNA, obtained by polymerase chain reaction, has been cloned into pTZ18R and pTZ19R. The resulting DNA sequence shows close homology with the bovine sequence. The derived amino-acid sequence shows conserved motifs similar to those observed in all species studied so far. No signal peptide is seen. Northern blots of RNA from lipopolysaccharide (LPS)-stimulated ovine alveolar macrophages show IL-1β m-RNA to be produced earlier than and to be more transient than IL-1α m-RNA. c-DNAs coding for the IL-1α proprotein and IL-1α and IL-1β mature proteins have been cloned and expressed in the yeast Ty-VLP system as fusion proteins. The resultant IL-1 protein preparations, cleaved from their fusion partners by the action of activated coagulation Factor Xa, are 80–95% pure and show biological activity in standard thymocyte co-mitogen and cartilage degradation assays for IL-1. Some species specificity is observed in that sheep thymoctyes are more responsive to ovine rIL-1 than are mouse thymocytes. The presence of a Factor Xa cleavage site in the IL-1α proprotein suggests that Factor Xa may be involved in the processing of ovine IL-1α to its mature form.

The cytokine interleukin 1 (IL-1) is an important endogenous mediator involved in regulation of immunological and inflammatory reactions, cellular activations and pathways of cellular maturation and differentiation. Either on its own or in conjunction with other mediators, IL-1 regulates the synthesis of other cytokines and is a potent activator of T cells and a lymphocyte promitogen. IL-1 has been reported to have actions on most cell types, either directly or by upregulating the expression of cellular receptors for other growth factors. It is involved in the maturation and activation of macrophages, bone marrow cell differentiation and cartilage and bone processing.[1–5] Epithelial cell growth has been reported to be inhibited but smooth muscle cell growth to be stimulated by IL-1.[6] Clinically IL-1 is involved in the progression of various disease states such as rheumatoid arthritis, inflammatory bowel disease, septic shock, tumourgenesis and in the pathogenesis of retroviral disease.[7–10] Many of the effects of IL-1 appear to oppose each other, not least because of concomitant production of specific inhibitors in many situations.[11,12] Recombinant IL-1 proteins are therefore very important in the elucidation and understanding of these actions and have possible therapeutic value.

Two distinct forms of IL-1 have been identified, α and β, having minimal sequence identity but each being well conserved across species.[13] The range of functions of the two proteins is similar. They also bind to different components of the same receptor(s) in a mutually exclusive manner.[12] Both proteins are initially produced as a proprotein of about 31 kDa which is subsequently cleaved to a mature 17–18 kDa form.[14–19] IL-1α is predominantly cell associated,[20] with small quantities only of the mature form being secreted. The proprotein and mature form are equally

From the Department of Veterinary Pathology, University of Edinburgh, Edinburgh EH9 1QH, Scotland.
*Author to whom correspondence should be addressed.
Received 4 March 1992; accepted for publication 23 March 1992

1043-4666/92/060418+11 $08.00/0

KEY WORDS: cloning/expression/factor Xa/interleukin 1/ovine

and Control, South Mimms, Herts, EN6 3QG, UK. Reference reagents for mouse TNF-α and human TNF-β are similarly available. Requests should be addressed to the Director.

active. On the other hand IL-1β in all species so far studied is secreted as the proprotein, which appears to require cleavage and consequent conformational change for both its activity and receptor binding.[11,12,14–18] Monocytes and polymorphonuclear leucocytes are capable of producing active IL-1β.[19,21] Fibroblasts and cultured human keratinocytes produce the inactive beta proprotein which is not processed.[18]

c-DNAs encoding the IL-1s have been sequenced from human, murine, bovine, porcine, rabbit and rat sources.[13,22–29] Recombinant human, murine and bovine mature IL-1 proteins expressed in bacterial and mammalian systems [13,20,22–27,30–31] have been reported to be active in a variety of in vitro assays such as the murine thymocyte co-mitogen, D10.G4.1 and EL4.NOB1.CTLL cell line assays.[32,33] The ovine IL-1β c-DNA sequence has been published.[34] We report here the cloning of ovine IL-1α c-DNA from alveolar macrophage RNA and the expression and characterization of ovine IL-1α and IL-1β in the yeast TY system. The recombinant proteins are 80–95% pure and are biologically active in standard IL-1 assays.

RESULTS

PCR, Sequencing and Cloning

c-DNA synthesized from polyA+ RNA from LPS-stimulated ovine alveolar macrophages was the substrate for all PCR reactions. From the ovine IL-Iβ sequence,[34] PCR primers were chosen as shown in Fig. 1, incorporating sequences required for the insertion into the yeast expression vector pOGS40 expression vector (Fig. 1B) of the c-DNA coding for the mature protein, IL-1β (mat).

To obtain the complete ovine IL-1α proprotein coding sequence, IL-1α(pro), primers were initially chosen from positions in the bovine RNA sequence which cover the whole coding region[13] and used in amplification of the same c-DNA populations. The resulting PCR product was cloned and sequenced. The ovine IL-1α c-DNA sequence is shown in Fig. 2. There is one potential *N*-linked glycosylation site, in the putative mature protein region. From the sequence a primer was chosen for the mature protein coding region IL-1α(mat). The amino terminus of the mature protein was assumed on the basis of human and bovine data.[13,22] All PCR products were sequenced, cloned into pTZ18R and pTZ19R and resequenced in both directions using single stranded DNA preparations. At least five independent clones for each form of IL-1 were sequenced. No sequence differences between individual clones or between the clones and the primary PCR products were seen.

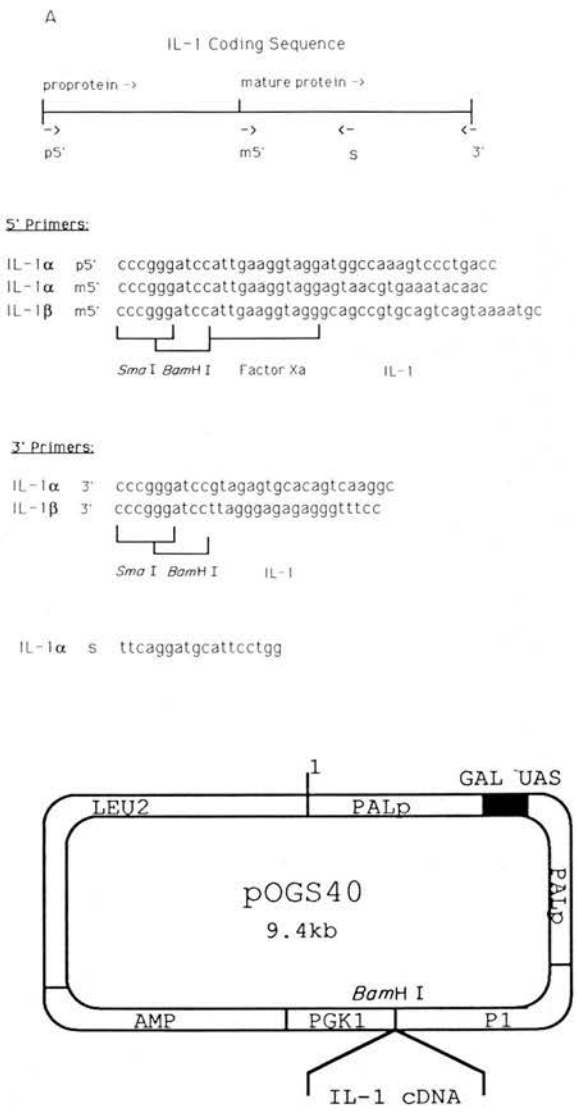


Figure 1. (A) PCR Primers. 5′ proprotein (p5′) and mature protein (m5′). (B) Map of expression vector pOGS40.

(A) PCR primers contain two enzyme sites, a Factor Xa site and the IL-1 sequence. 3′ primers (3′) have no Factor Xa site. IL-1α proprotein primers are from the bovine sequence.[13] IL-1α mature protein primers and s, which is an internal sequencing primer, were chosen from the resulting ovine PCR sequence. IL-1β primers are from our already published ovine sequence. (B) Map of cloning vector pOGS40 (42) which carries an AMP resistance gene for selection purposes. IL-1 c-DNA, containing its own terminator codon, is ligated into the vector using the unique *Bam*HI site. IL-1 fused to the yeast P1 protein is synthesised under control of the hybrid PGK/GALp promoter in galactose medium.

Homologies between IL-1 sequences of different species are shown in Table 1. There is only 25% identity between ovine IL-1α and β. Similar identities have been found between the IL-1α and IL-1β of bovine, human and murine IL-1s.[13,22,25] Subsequent to cloning the inserts into the pOGS40 vector, re-

```
atggccaaagtccctgacctctttgaagacctgaagaactgttacagtgaaaatgaagac   60
M  A  K  V  P  D  L  F  E  D  L  K  N  C  Y  S  E  N  E  D     20

tacagttctgaaattgaccacctctctctgaatcagaagtccttctatgatgcaagctat  120
Y  S  S  E  I  D  H  L  S  L  N  Q  K  S  F  Y  D  A  S  Y     40

gagccacttcgtgaggaccacatgaataagtttatgtccctggatacctcggaaacctcc  180
E  P  L  R  E  D  H  M  N  K  F  M  S  L  D  T  S  E  T  S     60

aagacatccaggcttagcttcaaggagaatgtggtgatggtgacagccaatggcaagatt  240
K  T  S  R  L  S  F  K  E  N  V  V  M  V  T  A  N  G  K  I     80

ctgaagaagagacggttgagtttaaatcagttcatcaccgatgatgacctggaagccatt  300
L  K  K  R  R  L  S  L  N  Q  F  I  T  D  D  D  L  E  A  I    100
                                                   V
gccaatgataccgaagaagaaatcatcaagcccagatcagcacattacagcttccagagt  360
A  N  D  T  E  E  E  I  I  K  P  R  S  A  H  Y  S  F  Q  S    120

aacgtgaaatacaactttatgagagtcatccaccaggaatgcatcctgaacgacgccctc  420
N  V  K  Y  N  F  M  R  V  I  H  Q  E  C  I  L  N  D  A  L    140

aatcaaagtataattcgagatatgtcaggtccatacatgacggctgctacattaaataat  480
N  Q  S  I  I  R  D  M  S  G  P  Y  M  T  A  A  T  L  N  N    160

ctggaggaggcagtgaaatttgacatggttgcttatgtatcagaagaggattctcagctt  540
L  E  E  A  V  K  F  D  M  V  A  Y  V  S  E  E  D  S  Q  L    180

cctgtgactctaagaatctcaaaaactcaactgtttgtgagtgctcaaaatgaagacgaa  600
P  V  T  L  R  I  S  K  T  Q  L  F  V  S  A  Q  N  E  D  E    200

cccgtcttgcataaggagatgcctgagacacccaaaatcatcaaagatgagaccaatctc  660
P  V  L  H  K  E  M  P  E  T  P  K  I  I  K  D  E  T  N  L    220

ctcttcttctgggaaaagcatgggtctatggactacttcaaatcagttgcccatccaaag  720
L  F  F  W  E  K  H  G  S  M  D  Y  F  K  S  V  A  H  P  K    240
                          ↑
ttgttcattgccacaaagcaagaaaaactggtgcacatggcaagcgggccgccctcgatc  780
L  F  I  A  T  K  Q  E  K  L  V  H  M  A  S  G  P  P  S  I    260

actgactttctgatattggaaaaatagccttgactgtgcactctac                826
T  D  F  L  I  L  E  K  *                                     280
```

Figure 2. Nucleotide and predicted amino acid sequence of ovine interleukin-1α.

DNA and protein sequences are numbered starting with the initiator Met. The presumed start of the mature protein is designated V. There is one potential *N*-glycosylation site, shown in bold print, in the mature protein.

sequencing confirmed the orientation and reading frame of the inserts.

m-RNA Production From Alveolar Macrophages Stimulated With LPS

Northern blot data (Fig. 3A,B) from LPS-stimulated ovine alveolar macrophages, suggest that ovine IL-1β m-RNA is produced earlier than IL-1α m-RNA, the β form showing a maximum at 4 h post-induction as opposed to 6 h for the α form. By 8 h the IL-1β mRNA is already declining and has all but disappeared by 24 h whereas the α message is still detectable. This is a similar profile of m-RNA production to that of human[19] and murine[10] IL-1s. Mixed IL-1α and IL-1β probe experiments suggest that more IL-1β than IL-1α m-RNA is produced (data not shown). This is in accordance with published data for bovine, murine and human IL-1s showing the β message to be produced in a 10 times greater abundance than the alpha. Ovine IL-1 m-RNAs MWs are *c.* 2.0 kb (α) and 1.6 kb (β), smaller than their bovine and human counterparts at *c.* 2.2 kb (α) and 2.0 kb (β).[13]

Protein Expression

pOGS40 is a galactose-inducible yeast expression vector which produces yeast P1 protein fused to the protein of interest. These fusion proteins assemble into virus-like particles (vlp) from which the inserted protein can be cleaved away by proteolysis with activated coagulation Factor Xa (FXa). P1 is released as vlp which can be removed by centrifugation. Selected colonies of yeast strain BJ2168 transfected with pOGS expression constructs were grown in liquid culture. Under induction conditions, 7×10^8 cells/ml were obtained for IL-1α(pro), 8×10^8 cells/ml for IL-1α(mat) and 7×10^8 cells/ml for IL-1β. Colonies constitutively expressing P1 (i.e. transfected with PMA5620) yielded *c.*10^9 cells/ml. Vlps were prepared as described in Materials and Methods. Electron micrographs of vlps show them to be somewhat irregular though basically circular and of varying sizes (data not shown).

TABLE 1. IL-1α and IL-1β – species comparisons. Figures show percentage similarities between ovine IL-1s and those from other species.

	Nucleic acid		Amino acid	
	α	β	α	β
Bovine	97	95	97	96
Human	81	76	72	77
Murine	80	70	72	56
Porcine	88	–	89	–
Rabbit	80	–	79	–

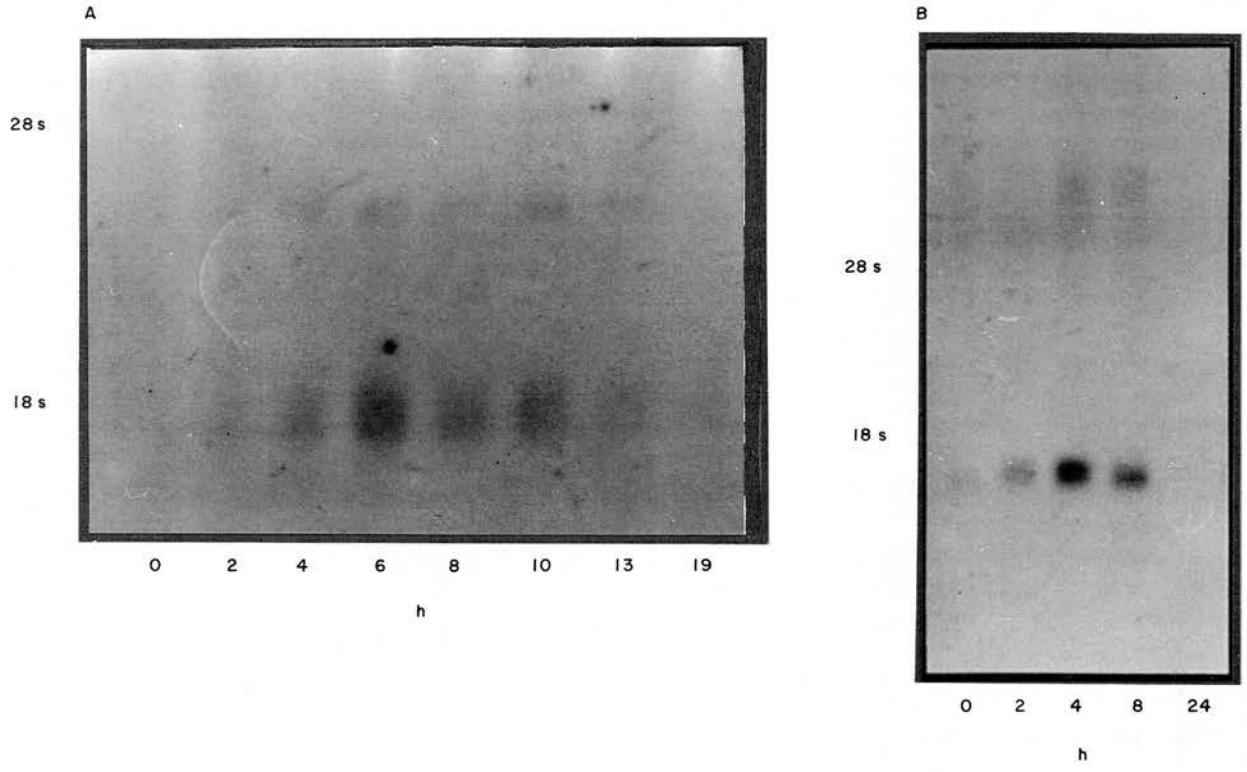


Figure 3. IL-1 gene expression in ovine alveolar macrophages. (A) IL-1α mRNA. (B) IL-1β mRNA.

10 μg total RNA extracted from ovine alveolar macrophages after stimulation with LPS for the times shown, was run on denaturing agarose gels and probed with ^{32}P-labelled full length IL-1α (A) or IL-1β (B) c-DNAs as probes (The 10 h time point, Fig. 3A, was slightly >10 μg as seen on the ethidium bromide stained gel prior to blotting). The positions of 18s and 28s RNA are shown for reference.

Figure 4 shows stages in the purification of the fusion proteins. Bands corresponding to the fusion proteins are clearly visible in extracts from yeast cells of the mature rIL-1 fusion proteins (Fig. 4A). Purified rIL-1β vlp preparations before and after cleavage with Factor Xa and the preparation of the rIL-1β mature protein (βm) after spinning out the vlps are shown in Fig. 4B. Also shown are the final preparations of rIL-1α pro- and mature proteins (αp, αm). The mobility of these proteins through the gels is consistent with the sizes expected for the fusion proteins (P1 protein travels at 50 kDa and when fused to the IL-1β mature protein should appear at about 68 kD). A western blot (Fig. 4C) probed with anti-P1 polyclonal antisera shows the presence of P1 in both the fusion protein and the P1 only preparations. Also shown is a FXa cleaved sample in which P1 (lane 2) is slightly truncated with respect to native P1 (lane 3). Yields of the purified recombinant proteins are: αm >150 μg/l of culture, αp >120 μg/l and βm >50 μg/l. These preparations are estimated to be 80–95% pure.

FXa cleavage of αp:P1 and βm:P1 fusion proteins gave rise to unexpected products. The predicted molecular weight of αp protein is about 31 kDa. However digestion of αp:P1 gave a main band running at about 21 kDa, just above that for the mature IL-1α protein. On digesting the βm vlp, two bands were consistently seen, but in varying proportions, running at about 20 kDa and 18 kDa, rather than just the one expected band at less than 20 kDa. On closer inspection of the amino acid sequences it appears that cleavage by the enzyme may occur at 'relaxed' sites (listed in Table 2). If the enzyme were to be cutting at these sites, proteins of the observed sizes would be produced. To test this possibility, time courses of digestion of the proteins were done with the results being shown in Fig. 5. It appears that the enzyme could indeed be cutting at the relaxed sites as the αp shows the expected 31 kDa band initially, with subsequent appearance of the 21 kDa band and eventual loss of the larger band (Fig. 5; see also Fig. 4B, lane 3). βm shows the initial appearance of the 20 kDa band followed by the 18 kDa band. On longer incubation the 20 kDa band disappears. The apparent molecular weight of the recombinant ovine IL-1β is larger than expected from the calculated size i.e. 20 kDa as opposed to 17.5 kDa. This is in good agreement with published data on other IL-1βs which exhibit the same phenomenon.[13]

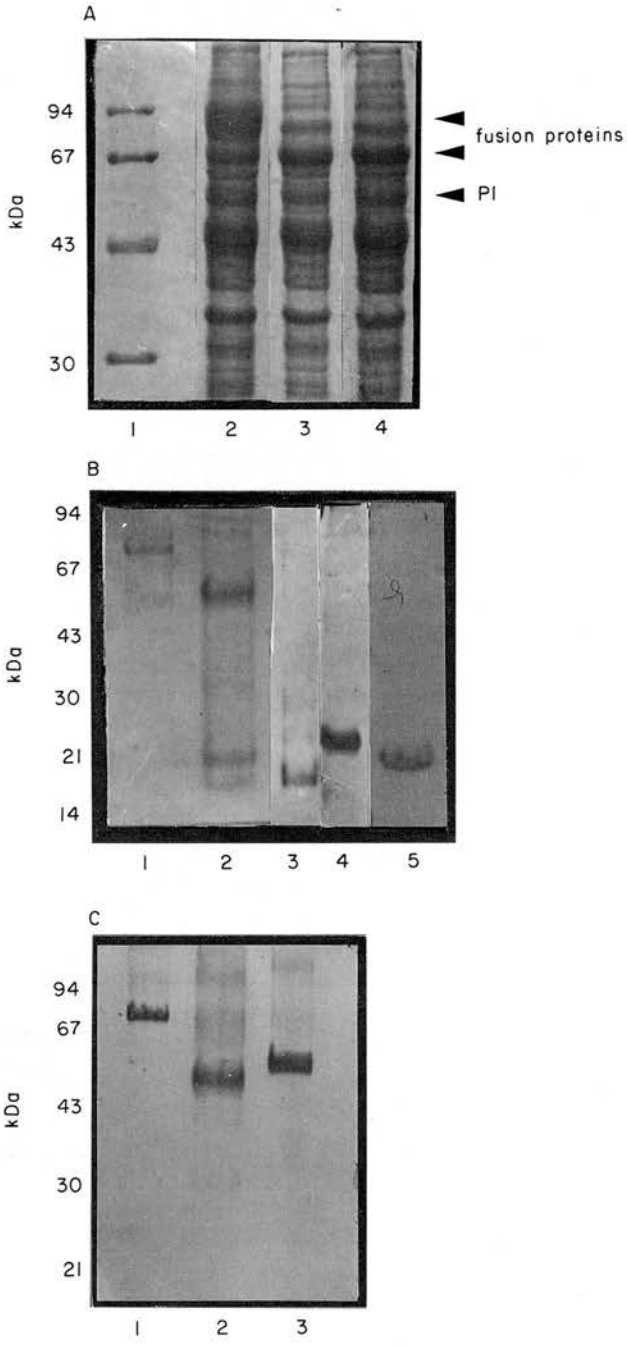


Figure 4. Purification of recombinant IL-1 from yeast.

Denaturing PAGE gels of: (A) Crude extracts of yeast cells containing fusion proteins run on a 10% gel and stained with Coomassie brilliant blue. Lane 2 shows proIL-1α:P1 (αp), lane 3 matIL-1α:P1 (αm) and lane 4, matIL-1β:P1 (βm). The positions of the fusion proteins and P1 vlps are denoted by arrows. (B) Purification of rIL-1s shown on 15% gels. Purified βm vlps (lane 1). FXa cut βm vlps (lane 2). FXa cleavage of αp and αm vlps, follow the same pattern and are not shown. Final matIL-1β product (lane 3). Final proIL-1α product (lane 4). Final matIL-1α product (lane 5). (C) Blotted 10% gel of purified mature IL-1β fusion protein (lane 1), Factor Xa cut fusion protein (lane 2) and yeast P1 protein (lane 3) hybridized with anti-P1 polyclonal antiserum showing the presence of P1 protein in the three species. Protein sizes are as indicated.

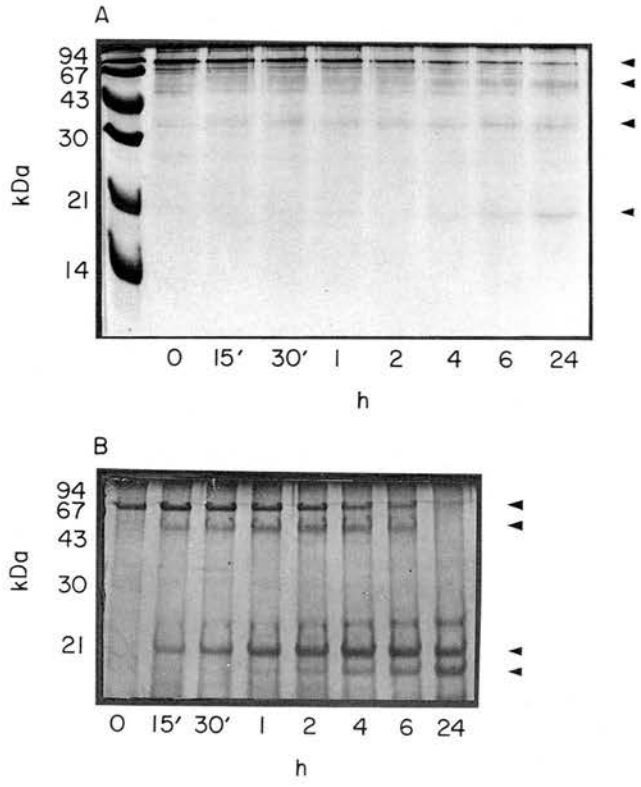


Figure 5. Cleavage of vlps by Factor Xa. (A) IL-1α proprotein, (B) IL-1β mature protein.

Time courses of the cleavage of IL-1 fusion proteins with 1:100 FXa (w/w). Products are shown run on 15% gels. (A) IL-1α proprotein on a 15% Coomassie blue stained gel. Bands of 81 kDa (fusion protein), 50 kDa (P1), 31 kDa (intact αp) and 20 kDa (αp final cleavage product) are labelled with arrows. (B) IL-1β mature protein on a 15% silver stained gel. Bands of 70 kDa (fusion protein), 50 kDa (P1), 20 kDa (βm) and 18 kDa (βm final cleavage product) are labelled with arrows (see also Fig. 4B, lane 4). The band at *c.* 25 kDa is a degradation product of P1 which appears after incubation 24 h at 25°C without enzyme.

TABLE 2. Factor Xa cleavage recognition sites. Motifs show peptide sequences required for cleavage by Factor Xa. Putative sites in ovine rIL-1s are shown in bold type.

Substrate	Peptide recognition sequence	Ref.
prothrombin	IDGR	43
β-globin	IEGR	44
HIV p24 vlp	ISPR	42
IL-1α	**IKPR** (position 110–113)	
IL-1β	**IEEK** (position 240–243)	

Biological Activity of Recombinant IL-1s

Induction of Thymocyte Proliferation

Figure 6A,B shows the results of incubating ovine thymocytes with mature rIL-1α or rIL-1β using 9 μg/ml PHA as co-mitogen. Using the accepted

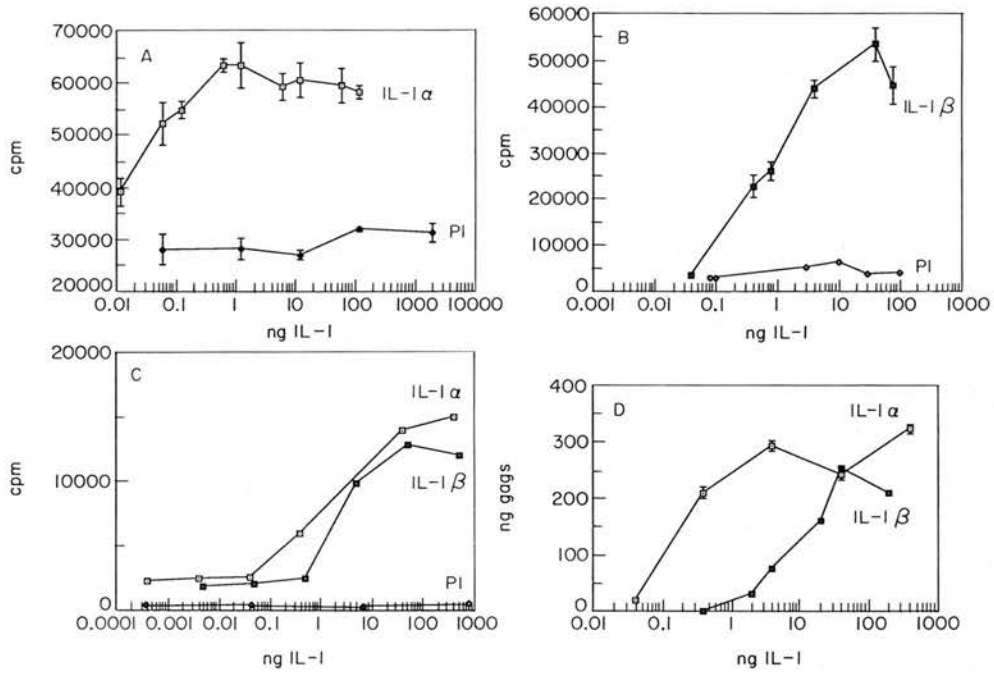


Figure 6. Biological assays for rIL-1 activity.

(A), (B) Sheep and (C) mouse thymocyte proliferation induced by rIL-1 with 9 μg/ml PHA as co-mitogen was measured by incorporation of ^{3}H-thymidine. (D) Degradation of sheep xiphoid cartilage by the action of rIL-1. Resultant glycosylaminoglycans (gags) were added to methylene blue dye and measured by the increase in absorbance at 540 μm as compared with a chondroitin sulphate standard curve. No release of gags by P1 was detected. In all assays, the P1 control is a preparation of constitutively expressed P1 vlps incubated with Factor Xa and containing yeast contaminants which could be present in the rIL-1 preparations. Concentrations were matched to the rIL-1 samples.

definition of activity (one unit being that amount of protein which yields half maximal counts per minute of incorporated ^{3}H-thymidine) one unit of ovine αm is 40pg and one unit of ovine βm is 800 pg in this assay. Samples assayed up to 5 weeks after cutting the vlps showed no decrease in their unit activity over this time if stored at 4°C. Aliquots of the same sample stored at −20°C show some loss of activity on freezing and incubation at 37°C for as little as 1 h caused a large loss of activity (data not shown). Purified porcine IL-1β was used as a comparison for activity. It is slightly more active than the ovine βm and less active than the ovine αm. Neither uncut IL-1 vlps nor a cut P1 protein control induce any thymocyte proliferation. There is considerable variation in activity from thymus to thymus which accounts for the differing levels of counts incorporated in the assays shown. About 50-fold higher amounts of IL-1α and 2.5-fold higher of IL-1β were required to stimulate murine thymocytes to proliferate (Fig. 6C).

Cartilage Degradation Assay

The rIL-1s were assayed for their action in triggering metalloprotease activity in ovine Xiphoid cartilage (Fig. 6C). The activities of both were about 10-fold less in this assay than in the ovine thymocyte assay, one unit of αm being 400 pg and of βm being 10 ng. As in the thymocyte assay, none of the controls affected the assay in any way.

DISCUSSION

An alignment between derived IL-1α amino-acid sequences of different species is shown (Fig. 7). As for all IL-1s described so far, no hydrophobic signal peptide is seen. Our IL-1α sequence is identical to that published very recently by Andrews et al.[35] except for one amino acid difference; Val compared to Met at position 73. Expression of IL-1 c-DNAs in the yeast TY system yielded recombinant proteins of 80–95% purity. The specificity of Factor Xa in cleaving these fusion proteins is relaxed, leading to the rIL-1α proprotein being truncated probably by loss of 113 amino acids from the amino terminal. By the same token the rIL-1β mature protein

on cleavage produces both the expected protein of 136 amino acids and a slightly smaller one probably of 113 amino acids, missing 23 amino acids from the carboxyl end. This cleavage is shown clearly by the time course of FXa cutting. In-vivo cleavage of the IL-1β from proprotein to active mature protein is thought to occur via the action of serine proteases. Potential processors include Proteinase K which has been shown by Hazuda et al.[17] to transform IL-1β to the active mature form which is Proteinase K insensitive, as is IL-1α. An endoproteinase, IL-1 converting enzyme[36,37] which appears to be found only in monocytes, has been shown to be able to cleave human and murine IL-1β specifically at Asp:Basic motifs (at positions 27 and 116) to yield an active protein. The ovine sequence contains this same motif and could potentially be processed similarly. Trypsin and chymotrypsin (cutting sites *ArgSer* and *PheLeu* respectively) have been proposed as IL-1α processors.[27,38] Of interest is that the putative sequence at which Factor Xa is cutting our IL-1α proprotein is IleuLysPro*ArgSer* (Fig. 7). Gln replaces Lys in the mouse but otherwise this sequence is present in all other species sequenced. Jones & Geczy[39] found that Factor Xa and thrombin greatly induced IL-1 activity in macrophages stimulated by suboptimal concentrations of LPS. It was proposed that this was via the action of FXa on prothrombin → thrombin because the effect of thrombin alone was greater than that of either FXa or prothrombin alone. From our results it appears that FXa at least, can act directly on ovine IL-1α. *N*-terminal analysis of purified natural IL-1α is needed to clarify the actual start of this protein. It remains to be seen whether the cut site in the mature IL-1β has any functional significance. Our data suggest that

Ovine IL-1α Sequence comparisons

```
Ovine     1 MAKVPDLFEDLKNCYSENEDYSSEIDHLSLNQKSFYDASYEPLREDHMDK
Bovine      ..................................................Q.N.
Human       ......M............ED..S............HV..G..H.GC..Q
Murine      ......................A................GS.H.TCT.Q
Porcine     ..................E...D...................PG.G...
Rabbit      ..............F....E...A...................H..C.N.

Ovine    51 FMSLDTSETSKTSRLSFKENVVMVTA---NGKILKKRRLSLNQFITDDDL
Bovine      .............K..........A.---S...................
Human       SV..SI.......K.T...SM.V.AT---...V.........S.S.....
Murine      .V..R......M.NFT...SR.T.S.TSS...........FSETF.E...
Porcine     ..P.S..K.......N..DS...AA.....................
Rabbit      VV..S.....VSPN.T.Q....A...---S................V..

                                    V
Ovine    97 EAIANDTEEEIIKPRSAHYSFQSNVKYNFMRVIHQEDILNDALNQSIIRD
Bovine      .....N.....................................
Human       ......S..........PF..L.........I.KY.F...........-
Murine      QS.TH.L..-T.Q....P.TY..DLR.KL.KLVR.KFVM..S...T.YQ.
Porcine     ................T......M........NHQC.............
Rabbit      .TNVS.P..G......VP.T..R.MR.KYL.I.K..FT........LV..

Ovine   147 MSGPYLTAATLNNLEEAVKFDMVAYVSE-EDSQLPVTLRISKYQLFVSAQ
Bovine      ........T..................-.............T.......
Human       ANDQ.....A.H..D.......G..K.SKD.AKIT.I.....T..Y.T..
Murine      VDKH..STTW..D.QQE.....Y..S.GGD..KY....K..DS.......
Porcine     P..Q..M..V....D.......A..T.N.D...........ETR......
Rabbit      T.DQ..R..P.Q..GD......G.....-...I.........QTP......

Ovine   197 NEDEPVLLKEMPETPKIIKD-ETNLLFFWEKHGSMDYFKSVAHPKLFIAT
Bovine      ..................-.............................
Human       D..Q............T.TGS.........T..TKN..T.....N.....
Murine      G..Q......L.......TGS..D.I...KSIN.KN..T.A.Y.E.....
Porcine     ..........L.....T...-..S.........N.......A.........
Rabbit      ..............R..T.S.SDI.....TQ.NKN....A...Q.....

Ovine   246 KQEKLVHMASGPPSITDFQILEK*
Bovine      ........................*
Human       ..DYW.CL.G............NQA*
Murine      .EQSR..L.R.L..M.....S*
Porcine     R........P.L..V.......NQS*
Rabbit      .P.H.....N.L..M.....S*
```

Figure 7. IL-1α amino acid alignments.

Amino acid translation products of published IL-1α sequences from various species are aligned to show conserved residues. The proposed start of the mature protein is designated V and a potential glycosylation site is in bold print. The proposed Factor Xa recognition site in ovine IL-1α is underlined.

both forms of IL-1β are active because preparations containing mainly 20 kDa or 18 kDa IL-1β are equally active in thymocyte and cartilage degradation assays (data not shown). This is not unexpected as amino acids 163–171 and 187–204 have been cited, from peptide studies, as being essential domains for activity in the mouse, each being important with respect to different functions of IL-1.[15] Deletion mutants have also been described,[40] requiring up to amino acid 259 for activity. Others have reported a core peptide which has retained biological activity.[41] Relaxed cutting sites of FXa are as in Table 2. It appears that in vitro the specificity for Factor Xa is not as rigid as has been reported.[43,44]

Both rIL-1α and β have been shown to be biologically active in a variety of assay systems (Fig. 6). The activity of αm on ovine thymocytes is × 10 greater than on ovine cartilage whereas βm is equally active on both cell types. Much data is currently emerging on the IL-1 receptors from which it is becoming clear that although the receptor will bind both forms of IL-1, it has at least two components, 80 and 100 kDa, with differing affinities for α and β. Differential expression of these on different cell types are probably implicated in the differing activities seen on assay. αp is active in all assays but this preparation is of course partially cleaved at a site close to the putative start of the mature protein. We are currently expressing αp in a mammalian system which should yield data on the activity of the intact proprotein. We have observed reduced activity of ovine rIL-1s on murine thymocytes. Similar species barriers have previously been seen between murine and human in various assays.[26,31]

In conclusion, therefore, we have cloned ovine IL-1α and IL-1β c-DNAs from alveolar macrophages. The encoded proteins expressed in yeast show biological activity in ovine assays as well as cross species activity in murine assays. The recombinant proteins can be produced fairly easily and in large quantities for further study of the actions and interactions of IL-1 in sheep diseases.

MATERIALS AND METHODS

Alveolar Macrophages

Macrophages obtained by lung lavage of gnotobiotic sheep were allowed to adhere to plastic culture bottles in Iscoves serum free medium at a density of 2×10^7 cells per bottle for 24 h prior to stimulation with 10 μg/ml lipopolysaccharide in fresh medium. At each of various time intervals up to 24 h post stimulation, total RNA was isolated from one bottle following the guanidine isothiocyanate method of Chirgwin et al.[45]

RNA Analysis

10 μg samples of total RNA were run on denaturing gels for northern blot analysis[46] of the time course of production of IL-1α and β m-RNAs. Blots were prehybridized for 4 h then hybridized with the appropriate ^{32}P-labelled probes for 20 h at 42°C in 5 × SSC, 0.5% low-fat dried milk, 1 mM EDTA, 0.5% SDS, 50% formamide and 125 μg/ml yeast RNA. Filters were washed with 2 × SSC, 0.1% SDS for 2 × 10 min at 22°C then 0.2 × SSC, 0.1% SDS for 3 × 10 min at 50°C.

PCR and Cloning of c-DNAs

First strand c-DNA synthesized from PolyA+ enriched RNA[47] was used as the template for PCR reactions as follows; denature 95°C, 30 s; anneal 52°C, 30 s; extend 72°C, 1.5 min; final extension 72°C, 5 min. The reaction buffer contained 100 μM dNTPs, 1.5 mM $MgCl_2$, 100 μg/ml BSA, 50 mM KCl, 3 mM DTT, 10 mM Tris pH8.8, 0.3 units Taq polymerase. PCR primers were as shown in Fig. 1. Initial IL-1α primers, to amplify the complete ovine IL-1α coding sequence were chosen from the bovine IL-1α sequence.[13] After sequencing the ovine IL-1α cDNA, we derived the primer sequence for the start of the mature protein as well as a primer, *s* in Fig. 1, for use as an internal sequencing primer. IL-1β primers for the mature coding region were from the previously published ovine IL-1β sequence.[34] A number of protective bases and a *Bam*H I site for ligation into the POGS40 vector were included in the primers. In addition, the sense primers contained a coding site for coagulation Factor Xa (FXa) recognition, to allow for cleavage of the expressed protein. PCR products were sequenced by the dideoxy chain termination method using Sequenase II.[48] For ligation into pTZ, the PCR products were made blunt ended by 'fill in' using Klenow fragment of DNA polymerse, phosphorylated, purified on 1.2% low gelling temperature agarose, the appropriate bands excised and ligated into pTZ18R and pTZ19R.[49] For sequencing, single-stranded DNA was synthesized from these constructs by the method of Messing.[50] For expression, inserts were excised from pTZ using *Bam*H I, gel purified and ligated into *Bam*H I digested pOGS40[42] expression vector (Fig. 2B). Blunt-ended PCR products were also digested with *Bam*H I, gel purified and ligated directly into pOGS40. Recombinant colonies were selected by colony hybridization using appropriate α^{32}P-dCTP-labelled PCR products.[51] CsCl gradient[52] purified plasmid was used for double-stranded sequencing of the recombinant pOGS vector and for transfection into yeast for protein expression.

Expression and purification of IL-1 proteins from yeast:

This method is essentially that of Gilmour et al.[42] with minor variations as detailed. pOGS40 constructs for inducible cytokine expression or pMA5620 for constitutive P1 expression, were transfected into yeast BJ2168 by spheroplasting, plating in regeneration agar (0.67% yeast nitrogen base; 1 M sorbitol; 1% glucose; 30% Difco agar) and incubating at 30°C. Colonies obtained, usually after 3–4 days, were restreaked onto 0.67% yeast nitrogen base

(YNB) plates and subsequently grown up in liquid culture. Yeast cultures for inducible expression were grown for 48 h at 30°C in YNB containing 1% glucose and 0.002% tryptophan then induced for 24 h with 1% galactose plus 0.3% glucose and tryptophan. Yeast cultures for constitutive expression were grown in YNB glucose medium, supplemented with 0.002% uracil. Cells were harvested, suspended in 10 mM Tris–HCl pH 7.4, 2 mM EDTA, 140 mM NaCl containing protease inhibitors and lysed by vortexing with acid washed glass beads. Supernatants were spun at 10 000 ***g***, the yeast debris discarded and the vlp containing suspension partially purified and concentrated by spinning at 30 000 ***g*** for 90 min onto a 60% sucrose cushion. The interface and cushion material were dialysed into 100 mM Tris pH7.4, 10 mM $CaCl_2$ containing 0.02% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate) and 0.02% DOC (NaDeoxycholate). Expressed protein was released from the vlps by digestion with Factor Xa (FXa) at a concentration of 1:100 w/w and further purified by spinning at 40 000 ***g*** to remove the P1 vlps present post cleavage as well as any remaining yeast proteins. Samples dialysed into PBS were stored at 4°C or −70°C.

Anti-P1 and Anti-FXa Antibodies

Rabbits were immunised with a P1 vlp preparation or FXa using standard techniques to obtain polyclonal antisera consisting of antibodies to either P1 plus yeast proteins or to FXa.

Analysis of Expressed Proteins

PAGE/SDS Gels

Vlps, cut vlps and isolated protein were analysed on polyacrylamide/SDS denaturing gels[53] stained with either 0.25% Coomassie brilliant blue or silver.[54] Blots of gels were incubated with 1/1600 anti-P1 first antibody, 1/8000 anti rabbit second antibody and developed with 0.2 mg/ml NBT (Nitrobluetetrazolium); 0.1 mg/ml BCIP (Bromo-chloro-imidolyl-phosphate toluidine salt); 0.2 mM $MgCl_2$ in 0.1 M Tris pH 9.5. Concentrations of recombinant proteins were estimated by comparing dilutions of each sample with protein standards on PAGE gels.

Thymocyte Co-stimulation Assay

Recombinant proteins were assayed using either mouse or sheep thymocytes at 1×10^6/ml with 9 μg/ml phytohaemagglutinin as co-mitogen. Samples were pre-incubated for 48 h at 37°C in 100 μl RPMI; 100 μl phosphate buffered saline; 5% fetal calf serum, then a further 16 h with 20 μCi ^{3}H-thymidine added. Cells were harvested and counted as a measure of de-novo DNA synthesis.

Cartilage Degradation Assay

This assay was performed as per Dingle et al.[55] with minor variations. Discs (2 mm × 1 mm) of fresh sheep xiphoid cartilage were incubated for 48 h in DME medium with 5% FCS prior to incubation for 24 h in fresh medium with the recombinant protein sample. Glycosaminoglycans released from the cartilage were measured by treating with methylene blue dye and comparing the absorbance at 540 mμ with a chondroitin sulphate standard curve.

EMBL Accession numbers are as follows: ovine IL-1 alpha X60167; ovine IL-1 beta X54796.

Acknowledgements

We would like to thank Dr Sally Adams of British Biotechnology Ltd. for components of the yeast TY expression system and Dr J Saklatvala for the porcine IL-1β. This work was supported by the Agriculture and Food Research Council (Grant No. AG15/329), by a Wellcome Trust project development award and by the Scottish Home and Health Department.

REFERENCES

1. Bartolini G, Orlandi M, Chiricolo M, Licastro F, Zambonelli P, Minghetti L, Tomasi V (1990) Interleukins and interferons: yin-yang modulators of PGH synthetase in human macrophages. Biofactors 2:267–270.

2. Kobayashi M, Imamura M, Gotohda Y, Maeda S, Iwasaki H, Sakurada K, Kasai M, Hapel AJ, Miyazaki T (1991) Synergistic effects of interleukin-1β and interleukin-3 on the expansion of human hematopoetic progenitor cells in liquid cultures. Blood 78:1947–1953.

3. Bayliss MT (1990) Proteoglycan structure and metabolism during maturation and ageing of human articular cartilage. Biochem Soc Trans 18:799–802.

4. Thomson BM, Saklatvala J, Chambers TJ (1986) Osteoblasts mediate interleukin 1 stimulation of bone resorption by rat osteoclasts. J Exp Med 164:104–112.

5. Mundy GR (1991) Inflammatory mediators and the destruction of bone. J Periodont Res 26:213–217.

6. Sawada H, Kan M, McKeehan WL (1990) Opposite effects of monokines (interleukin-1 and tumour necrosis factor) on proliferation and heparin binding (fibroblast) growth factor binding to human aortic endothelial and smooth muscle cells. In Vitro Cell Dev Biol 26:213–216.

7. Kirkham B (1991) Interleukin-1, immune activation pathways and different mechanisms in osteoarthritis and rheumatoid arthritis. Ann Rheum Dis 50:395–400.

8. di Giovine FS, Duff GW (1990) Interleukin 1: The first interleukin. Immunol Today 11:13–20.

9. Meltzer MS, Skillman DR, Hoover DL, Hanson BD, Turpin JA, Kalter C, Gendelman HE (1990) Macrophages and the human immunodeficiency virus. Immunol Today 11:217–223.

10. Chensue SW, Terebuh PD, Remick DG, Scales WE, Kunkel SL (1991) In vivo biologic and immunohistochemical analysis of interleukin-1 alpha, beta and tumor necrosis factor during experimental endotoxemia. Kinetics, Kupfer cell expression and glucocorticoid effects. Am J Pathol 138:395–402.

11. Dayer JM (1991) Chronic inflammatory joint diseases: natural inhibitors of interleukin 1 and tumor necrosis factor alpha. J Rheumatol Suppl 27:71–75.

12. Dinarello CA (1991) Interleukin-1 and interleukin-1 antagonism. Blood 77:1627–1652.

13. Malisewski CR, Baker PE, Schoenborn MA, Davis BS, Cosman D, Gillis S, Cerretti DP (1988) Cloning, sequencing and expression of bovine interleukin 1α and interleukin 1β complementary cDNAs. Mol Immunol 25:429–437.

14. Beuscher H, Gunther C, Rollinghof M (1991) IL-1 beta is secreted by activated murine macrophages as biologically inactive precursor. J Immunol 144:2179–2183.

15. Boraschi D, Tagliabue A (1990) Human interleukin 1: structure-function relationship. Ann Ist Super Sanita 26: 273–282.

16. Hazuda DJ, Lee JC, Young PR (1988) The kinetics of Interleukin 1 from activated monocytes. Differences between interleukin 1α and interleukin 1β. J Biol Chem 263:8473–8479.

17. Hazuda DJ, Strickler J, Simon P, Young PR (1991) Structure-function mapping of interleukin 1 precursors. Cleavage leads to a conformational change in the mature protein. J Biol Chem 266:7081–7086.

18. Mizutani RB, Kupper TS (1991) Human keratinocytes produce but do not process pro-interleukin-1 (IL-1) beta. Different strategies of IL-1 production and processing in monocytes and keratinocytes. J Clin Invest 87: 1066–1071.

19. Bernaudin JF, Yamauchi K, Wewers MD, Tocci MJ, Ferrans VJ, Crystal RG (1988) Demonstration by in situ hybridisation of dissimilar IL-1 beta gene expression in human alveolar macrophages and blood monocytes in response to lipopolysaccharide. J Immunol 140:3822–3829.

20. Fuhlbrigge RC, Fine SM, Unanue ER, Chaplin (1988) Expression of membrane interleukin 1 by fibroblasts transfected with murine pro-interleukin-1 alpha cDNA. Proc Natl Acad Sci USA 85:5649–5653.

21. Mori S, Goto S, Okhawara S, Maeda K, Shimada K, Yoshinaga M (1988) Cloning and sequence analysis of a cDNA for lymphocyte proliferation potentiating factor of rabbit polymorphonuclear leukocytes: identification as a rabbit interleukin-1β. Biochem Biophys Res Commun 150:1237–1243.

22. March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, Gillis S, Henney CS, Kronheim SR, Grabstein K, Conlon PJ, Hopp TP, Cosman D (1985) Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature 315:641–648.

23. Auron PE, Webb AC, Rosenwasser LJ, Mucci SF, Rich A, Wolff SM, Dinarello CA (1984) Nucleotide sequence of human monocyte interleukin-1 precursor cDNA. Immunol 81:7907–791.

24. Furutani Y, Notake M, Yamaoshi M, Yamagishi J, Momura H, Ohue M, Furuta R, Fukui T, Yamada M, Nakamura S (1985) Cloning and characterisation of the cDNAs for human and rabbit interleukin-1 precursor. Nucleic Acids Res 13:5869–5882.

25. Gray PW, Glaister D, Chen E, Goeddel DV, Pennica D (1986) Two interleukin 1 genes in the mouse: cloning and expression of the c-DNA for murine interleukin 1β. J Immunol 137:3644–3648.

26. Huang JJ, Newton RC, Rutledge SJ, Horuk R, Matthew JB, Covington M, Lin Y (1988) Characterisation of murine IL-1 beta. Isolation, expression and purification. J Immunol 140:3838–3843.

27. Lomedico PT, Gubler U, Hellman CP, Dukovich M, Giri JG, Pan Y-C, Coller K, Seminow K, Chua AO, Mizel SB (1984) Cloning and expression of murine interleukin-1 cDNA in *Escherichia coli*. Nature 312:458–462.

28. Malisewski CR, Renshaw BR, Schoenborn MA, Urban JF, Baker PE (1990) Porcine IL-1α cDNA nucleotide sequence. Nucl Acids Res 18:4282.

29. Nishida T, Hirato T, Nishino N, Mizuno K, Sekiguchi Y, Takano M, Kawai K, Nakai S, Hirai Y (1988) Cloning of c-DNAs for rat interleukin-1 alpha and beta. Prog in Leukocyte Biol 8:73–78.

30. Kronheim SR, Cantrell MA, Deeley MC, March C, Glakin P, Anderson T, Hemenway T, Merriam J, Cosman D, Hopp T (1986) Purification to homogeneity of IL-1 beta protein. Biotechnology 4:1078–1082.

31. Tocci MJ, Hutchinson NI, Cameron PM, Kirk KE, Norman DJ, Chin J, Rupp EA, Limjuco GA, Bonilla-Argudo VM, Schmidt JA (1987) Expression in *Escherichia coli* of fully active recombinant human IL-1 beta: comparison with native human IL-1 beta. J Immunol 138:1109–1114.

32. Kaye J, Procelli S, Tite J, Jones B, Janeway CA (1983) Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen-presenting cells in the activation of T cells. J Exp Med 158:836–856.

33. Le Moal MA, Stoeck M, Cavaillon JM, MacDonald HO, Truffa-Bachi P (1988) A sensitive, IL-2 dependent, assay for IL-1. J Immunol Methods 107:23–30.

34. Fiskerstrand C, Sargan D (1990) Nucleotide sequence of ovine interleukin-1 beta. Nucl Acids Res 18:1765.

35. Andrews AE, Barcham GJ, Brandon MR, Nash AD (1991) Molecular cloning and characterisation of ovine IL-1α and IL-1β. Immunology 74:453–460.

36. Howard AD, Kostura MJ, Thornberry N, Ding GJF, Limjuco G, Weidner JPS, Hogquist KA, Chaplin DD, Mumford RA, Schmidt JA, Tocci MJ (1991) IL-1 converting enzyme requires aspartic acid residues for processing of IL-1β precursor at two distinct sites and does not cleave the 31-kDa IL-1α. J Immunol 147:2964–2969.

37. Mosley B, Dower S, Gillis S, Cosman D (1987) Determination of the minimum polypeptide lengths of the functionally active sites of human interleukins-1 α and β. Proc Natl Acad Sci USA 84:4572–4576.

38. Cameron PM, Limjuco GA, Chin J, Silberstein L, Schmidt JA (1986) Purification to homogeneity and amino acid sequence analysis of two anionic species of human interleukin-1. J Exp Med 164:237–250.

39. Jones A, Geczy CL (1990) Thrombin and Factor Xa enhance the production of interleukin-1. Immunol 71:236–241.

40. Mosley B, Urdal D, Prickett K, Larsen D, Cosman D, Conlon P, Gillis S, Dower SK (1987) The interleukin 1 receptor binds the human interleukin-1α precursor but not the interleukin-1β precursor. J Biol Chem 262:2941–2944.

41. Schmidt JA, Bomford R (1991) The processing of interleukin-1 beta studied with antibodies raised against synthetic peptides from the precursor N-terminal region. Cytokine 3:240–245.

42. Gilmour JEM, Senior JM, Burns NR, Esnouf MP, Gull K, Kingsman SM, Kingsman AJ, Adams SE (1989) A novel method for the purification of HIV-1 p24 protein from hybrid Ty virus-like particles (Ty-VLPs). AIDS 3:717–723.

43. Magnusson S, Petersen TE, Sottrup-Jensen L, Cleays H (1975) In Reich E, Rifkin D, Shaw E, (eds) Proteases and biological control. Cold Spring Harbor Laboratory, New York, pp 123–149.

44. Nagai K, Thogersen HC (1984) Generation of β-globin by sequence-specific proteolysis of a hybrid protein produced in *E. coli*. Nature 309:810–812.

45. Chirgwin JM, Przybyla AE, MacDonald RJ, Ruffer WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299.

46. Thomas PS (1980) Hybridisation of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci 77:5201–5205.

47. Gubler U, Hoffman BJ (1983) A simple and very efficient method for genrating cDNA libraries. Gene 25:263–269.

48. Tabor S, Richardson CC (1987) DNA sequence analysis with a modified bacteriophage T7 polymerase. Proc Natl Acad Sci USA 84:4767–4771.

49. Mead DA, (1986). Protein Engineering 1:67–70.

50. Messing J (1983) New M13 vectors for cloning. Methods in Enzymol 101:27–78.

51. Feinberg AP, Vogelstein B (1983) Technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13.

52. Manniatis T, Fritsch EF, Sambrook J (1982). Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, New York, pp 90–93.

53. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.

54. Morrisey JH (1981) Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem 117:307–310.

55. Dingle JT, Saklatvala J, Hembry R, Tyler J, Fell HB, Jubb R (1979) A cartilage Catabolic Factor from Synovium. Biochem J 184:177–180.